IMMUNE RESPONSES TO INGESTED PROTEIN ANTIGENS IN MICE

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For Douglas

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I declare that this thesis was composed by myself and that unless otherwise specified, the work was performed personally while I was part of a research team.

ABSTRACT OF THESIS

Immunological consequences and the regulation thereof, of antigen when introduced into the gastrointestinal tract of mice was examined in this thesis. A protocol of feeding 25 mg of a soluble protein antigen ovalbumin to mice which were immunologically naive with respect to this antigen, was confirmed as a means of inducing systemic hyporesponsiveness. Antigen-stimulated lymphocyte transformation in vitro was then tested as a means of exploring the development of cell-mediated immune responses in the mesenteric and peripheral lymph nodes of mice fed with ovalbumin: after a brief, generalised low-grade response occurring one day after feeding, results suggested that regulatory cells are present in mesenteric lymph nodes at between five and seven days following ovalbumin ingestion which prevent the initiation of a local cell-mediated immune response at this time.

The main emphasis of the thesis was concerned with the role of the antigen in inducing hyporesponsiveness. An aspect of intestinal function, presenting antigen as a tolerogenic stimulus to the systemic immune system following antigen feeding, was examined in serum transfer experiments: antigen transferred with serum, which was collected from mice one hour after a single feed of 25 mg ovalbumin, induced tolerance for delayed-type hypersensitivity when injected into naive syngeneic recipients and was called gut-processed antigen. Gut-processed ovalbumin had no effect on systemic humoral immunity.

The suppressive effect of gut-processed ovalbumin on systemic cell-mediated immunity was not antigen dose-dependent but seemed rather the results of structural alteration to the antigen during gut-processing. Immunoadsorption and gel filtration techniques

revealed that gut-processed ovalbumin possessed antigenic structures for anti-ovalbumin antibody and had an estimated molecular weight similar to native ovalbumin. Structural alteration of the antigen to become a tolerogen is therefore of a subtle nature.

Immuno-compromised mice treated with cyclophosphamide were not tolerized by injection with gut-processed ovalbumin which suggests that the tolerogen exerts its regulatory effect by activating suppressor cells.

Mucosal integrity and perhaps also an intact GALT are important in the intestinal processing of ovalbumin since tolerogen production was prevented by radiation damage in the intestine but could be restored by injecting lymphoid cells shortly after radiation.

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PUBLICATIONS

The following publications have arisen from work described in this thesis:

Strobel, S., Mowat, A. McI., Drummond, H. E., Pickering, M. G., Ferguson, A. Oral tolerance for CMI is due to activation of cyclophosphamide-sensitive cells by gut-processed antigen. Immunology 1983; 49: 451 - 456.

Mowat, A. McI., Strobel, S., Pickering, M. G., Drummond, H. E., Ferguson, A. Evidence that tolerance of cell-mediated immunity in mice fed ovalbumin is due to suppressor cells activated by intestinally-derived protein moities. Ann NY Acad Sci 1983; 409: 853 - 854.

LIST OF ABBREVIATIONS

Ab	antibody
abs	absorbed
BSA	bovine serum albumin
C	complement
Ci	curie
CMI	cell-mediated immunity
cpm	counts per minute
CY	cyclophosphamide
Den	denatured
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immuno-sorbent assay
F	gel filtration fraction
FCA	Freund's complete adjuvant
GALT	gut-associated lymphoid tissue
hr	hour
HSA	human serum albumin
Ia	immune-associated antigen
i.d.	intradermal
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
i.g.	intragastric
i.p.	intraperitoneal
i.v.	intravenous
Kav	partition coefficient
LTT	lymphocyte transformation test
Ly	mouse T lymphocyte associated antigen

M cell	microfold cell
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MW	molecular weight
n	number
neg	negligible
NK cell	null killer cell
NMS	normal mouse serum
NS	not significantly different
OD	optical density
OVA	ovalbumin
P	probability
PBS	phosphate buffered saline
PFC	plaque-forming cell
PHA	phytohaemagglutinin
PLN	peripheral lymph node
r	correlation coefficient
R, rad	radiation absorbed dose
S4B-anti-OVA	anti ovalbumin IgG covalently coupled to Sepharose 4B
sIgA	secretory IgA
sal	saline
s.d.	standard deviation
s.e.	standard error of mean
T_H	helper T lymphocyte
T_S	suppressor T lymphocyte
Thy 1.2	mouse T lymphocyte alloantigen
Vo	void volume
Ve	elution volume

Vt	total volume (gel filtration column)

Length

cm	centimetre
mm	millimetre
µm	micrometre
nm	nanometre

Volume

l	litre
ml	millilitre
µl	microlitre

Weight

kg	kilogram
g	gram
mg	milligram
µg	microgram
ng	nanogram

Concentration

M	molar
mM	millimolar
N	normal

Miscellaneous

g	gravitational acceleration
$\log_{10}$	logarithm to the base 10

pH	reciprocal $\log_{10}$ hydrogen ion concentration

Symbols

<	less than
=	equal to
/	per
↯	irradiated

INTRODUCTION TO EXPERIMENTS

Antigens introduced into the gastrointestinal tract are known to cause quite different immune responses to those observed when the same antigens are injected parenterally. For example, specific systemic immune tolerance is often the response to fed antigen and this can occur with the concomitant induction of local mucosal immunity. A growing interest has developed in the T lymphocyte - mediated regulation of responses to antigen feeding following the identification of different sub-populations of T lymphocytes and their functional characterisation.

It was already known for many years, that the form in which an antigen was administered could influence the immune response to that antigen. For example, deaggregated proteins are often highly tolerogenic. Chemical modification of antigen can either increase or decrease immunogenicity depending on how the antigenic structure is altered. More recently, it has been shown that B lymphocytes or different T lymphocyte populations are preferentially activated depending on the form of antigen encountered in an immune reaction.

The subject of my research therefore, has been antigen itself, in the context of changes which may occur in an antigen which is administered via the gastrointestinal tract as opposed to a parenteral injection and concerned mainly with the outcome of such antigen encounters seen in the systemic immune responses.

I chose the soluble protein ovalbumin as a model antigen for use throughout the study. Antigen which had been absorbed into the circulation from the intestines of mice was collected in the serum one hour after feeding. The antigen in serum was called gut-processed antigen and its immunological properties were defined by various in

in vitro and *in vivo* assays.

Fairly standard experiments were first of all performed in order to confirm the outcome of antigen feeding under controlled conditions of antigen dosage and systemic tolerance. Once this was achieved, the next stage in my investigation was to determine the effect of antigen feeding on the activation of T lymphocytes *in vitro* after parenteral and gastrointestinal exposure to antigen. These experiments met with only limited success and since parallel experiments on gut-processed antigen were proving more fruitful, I took up this latter line of investigation more fully. Therefore, serum was collected from mice one hour after a first-time exposure to ovalbumin introduced into the gastrointestinal tract and then tested in order to answer several questions about the nature of antigen after its absorption from the gut:

What are the in vivo immunological effects of gut-processed antigen?

A biological assay was developed in order to attempt to answer this question involving the transfer of serum from mice fed ovalbumin, into syngeneic recipients which were later immunized and challenged in order to assess their systemic immunity.

Does gut-processed antigen activate cells in vitro?

In a pilot study, some serum containing gut-processed ovalbumin was added to lymphocytes in a transformation test.

How much antigen is present in serum one hour after feeding?

This question was answered by testing serum in an ELISA which measured ovalbumin concentration.

From previous work, it was already known that pretreatment of mice with the drug cyclophosphamide abrogates orally-induced tolerance. Was this effect due to the action of cyclophosphamide on gut-processing or on T lymphocytes, in particular, T suppressor cells which are known to be sensitive to cyclophosphamide?

Both of these possibilities were explored in a series of serum transfer experiments, where either serum donors were injected with cyclophosphamide before feeding to investigate its effect on antigen processing by the gut, or where serum recipients were treated with cyclophosphamide before transfer of serum in order to examine effects on T suppressor cells.

These experiments led to further work to examine whether immunologically compromised hosts would be affected in their ability to process fed antigen. A lethal dose of total body irradiation was given to mice to produce compromised hosts which were used as donors in serum transfer experiments. Histological examination of the intestines of these donors obtained at post mortem, confirmed severe gut damage with lymphoid depletion, and the effect of this on antigen processing was tested in the recipients of serum from these mice.

Can gut-processing be reproduced by parenteral injection of modified antigen?

This question was addressed by giving groups of mice parenteral injections of native, deaggregated or urea-denatured ovalbumin over a range of doses based on those concentrations measured in the serum after ovalbumin feeding. The mice then had their systemic immunity assessed by a standard protocol.

Exposure to antigen can produce different effects on the humoral and cell-mediated limbs of the immune response. Therefore, in my studies on immune recognition of gut-processed antigen, I investigated whether there might be differences in determinants recognised by B cells (via surface antibody) and by T cells (via T cell receptors). This was approached by subjecting serum from antigen-fed mice to a variety of immunochemical techniques such as immunoadsorption to remove antibody binding sites followed by bioassay in the serum transfer system. Gel filtration was used as a means of obtaining gut-processed antigen fragments within defined molecular weight limits for testing within the serum transfer protocol. The results of these experiments served to further elucidate the nature of gut-processed ovalbumin both as an antigen, and as a protein entity whose molecular size could be estimated.

CHAPTER 1

GENERAL INTRODUCTION

1.1 The Immunological Apparatus of the Intestine

Within the gastrointestinal tract are many areas of organised lymphoid tissue such as the Peyer's patches. There are also cells dispersed under and within the mucosal epithelium which are capable of taking part in specific immune reactions.

In addition to specific immunity, various types of cells, including mononuclear phagocytes and mucosal mast cells, and physiological factors such as gastric acid, lysozyme and peristalsis enhance non-specific immunity in the gut (Ferguson and Mowat, 1980). Under certain conditions, mucosal mast cells can release histamine and cause increased secretion of mucus by goblet cells. Mucus, which coats the epithelial surface of the gut and forms an important protective covering can prevent the attachment of antigens to the epithelium and even facilitate their clearance from the intestinal lumen, (Woloschak and Tomasi, 1983). Gut epithelial cells are being continuously exfoliated into the lumen and so non-specific immunity is also maintained by the continued renewal of the absorptive surface of the intestine (Ferguson and Mowat, 1980).

1.2 Gut-Associated Lymphoid Tissue

A. PEYER'S PATCHES

Distributed throughout the epithelial membrane of the gastrointestinal tract, Peyer's patches can be seen as visible, non-encapsulated nodules of lymphoid tissue containing predominantly lymphocytes. The size and number of Peyer's patches varies with age

(in humans), size increasing with age, whereas the total number of Peyer's patches decreases from puberty after an initial increase from birth. Each Peyer's patch contains a variable number of lymphoid follicles and is one follicle thick (Cornes, 1965).

From studies using electron microscopy, Peyer's patches appear smooth and round, projecting into the gut lumen. Some of the epithelial cells covering Peyer's patches are known as "microfold" or M cells because of the many-folded ridges on their surfaces (Owen and Jones, 1974). M cells possess many intracellular vesicles which are thought to be used by the M cell in the transport of antigenic material from the gut to the underlying lymphoid cells, where the sensitisation of lymphocytes can occur (Woloschak and Tomasi, 1983).

B. INTRAEPITHELIAL LYMPHOCYTES

Intraepithelial lymphocytes are found, as their name suggests, between adjacent mucosal epithelial cells and concentrated in the basal part of the villus epithelium (Ferguson, 1977). They vary in their distribution along the gastrointestinal tract and increase in number during certain disease processes (Ferguson, 1976). The number of intraepithelial lymphocytes is also influenced by age, microbial antigens, parasitic infections, irradiation and immunosuppressive drugs (Ferguson, 1977). Intraepithelial lymphocytes can cross the basement membrane in both directions. They are generally larger than peripheral blood lymphocytes and can contain cytoplasmic granules many of which resemble mast cell granules (Woloschak and Tomasi, 1983; Ferguson, 1977).

Different types of cells have been identified as intraepithelial lymphocytes (Woloschak and Tomasi, 1983). For example the OKT 8

antigen associated with suppressor/cytotoxic function, has been found on 70% of human intraepithelial T lymphocytes. Both T and B cells have been identified in the intraepithelial lymphocyte populations of experimental animals and a recent study in mice (Mowat et al, 1983), reports the presence of intraepithelial lymphocytes with null-killer (NK) activity together with the presence of NK-regulating cells. Other intraepithelial cells which have been observed include eosinophils and globule leukocytes or mast cells (Collan, 1977).

C. CELLULAR TRAFFIC IN GUT-ASSOCIATED LYMPHOID TISSUE

In many different experiments, small T and B lymphocytes have been seen to "traffic" or migrate from the bloodstream into the Peyer's patches as well as to the lymph nodes and spleen. However, these small lymphocytes do not "home" to the intestinal mucosa. In contrast, studies on mice, rats and rabbits have shown that after sensitisation by antigen, B lymphocytes in Peyer's patches divide to become lymphoblasts which then migrate via the lymphatics to exocrine tissues such as the mammary and salivary glands (Woloschak and Tomasi, 1983), where they differentiate into plasma cells secreting IgA.

Thoracic duct lymphocytes are not the only source of lamina propria lymphocytes as seen when thoracic duct lymphocytes were diverted from the gut: whilst the number of IgA-plasma cells decreased in the lamina propria, it was not depleted of cells (Woloschak and Tomasi, 1983). This suggests that either some other source of plasma cells is capable of populating the lamina propria or that plasma cell numbers are maintained by local cell division. This idea is supported by experiments which showed that lamina propria cells can re-populate irradiated recipients in cell transfer

experiments (Woloschak and Tomasi, 1983).

The migration of IgA-plasma cell precursors to the lamina propria occurs independently of gut antigen, but the presence of antigen influences the number and distribution of migrating cells (Woloschak and Tomasi, 1983: Ferguson, 1978), a greater density of plasma cells being found at the site of antigen challenge (Pierce and Gowans, 1975).

There is, as yet, no established mechanism to explain the localisation of IgA precursors in the gut tissues. One model proposes a receptor for IgA precursors within the vascular compartment of the gut, another, that lymphoblasts migrate into the gut from the circulation at random, but that IgA-committed cells have an enhanced ability to proliferate in mucosal tissues (Woloschak and Tomasi, 1983).

Experimental evidence is still lacking for an explanation of the regulation of lymphoblast homing to the gut although it seems that there is a relationship between lymphoblast accumulation in a segment of gut, and the regional blood flow to that area (Ottaway and Parrot, 1980). Hormones can influence lymphocyte homing to mucosal tissues (Bienenstock and Befus, 1980), and T cells may play a role in the selective retention of B lymphoblasts in the lamina propria since T cells of mesenteric lymph nodes home to the gut (Guy-Grand et al, 1978).

In addition to the homing of lymphoblasts from lymph to lamina propria, animal experiments have confirmed the migration of cells from lymph to the intraepithelial site (Guy-Grand et al, 1974: Parrot et al, 1975). Lymphocytes destined to enter the epithelium leave the bloodstream via capillaries in the villi. The route by which

intraepithelial lymphocytes leave the epithelium is not yet established, but it is thought likely that these cells re-enter the lamina propria and leave the villi in the lymphatics (Ferguson, 1977).

D. ANTIGEN PRESENTING CELLS IN GUT-ASSOCIATED LYMPHOID TISSUES

Various antigen presenting cells have been described in Peyer's patches and the intestinal lamina propria. These include macrophages (Richman et al, 1981; Mayrhofer et al, 1983), and also non-phagocytic dendritic cells which bear Ia antigen on their surface (Mayrhofer et al, 1983). These latter cells are found in the lamina propria of the villi and crypts and in Peyer's patches, both in the sub-epithelial region and within the epithelium itself. In addition to these accessory cells, the gastrointestinal epithelial cells can express Ia antigens on their surfaces (Mayrhofer et al, 1983), a marker associated with antigen presenting ability, and thus suggesting a possible role for the gut epithelium in local antigen presentation.

1.3 The Fate of Protein Entering the Gastrointestinal Tract

The immunological properties of the small intestine cannot be considered in isolation from the digestive properties of this complex organ. Digestion and absorption of both endogenous and exogenous (dietary) protein is one of the principal functions of the gastrointestinal tract and is an extremely efficient process (Reviewed by Freeman and Kim, 1978).

A. PROTEIN DIGESTION IN THE GASTROINTESTINAL TRACT

Protein digestion is initiated in the stomach when the major

gastric protease, pepsin, is activated from pepsinogen. Gastric phase digestion is influenced by hormones and other, nonhormonal factors such as gastric acid and rate of gastric emptying. Gastric phase protein digestion gives way to intestinal digestion by proteases released by the pancreas.

Digestion by pancreatic enzymes is more critical than the gastric phase of digestion. Activated pure pancreatic juice contains both endopeptidases and exopeptidases, and the products of their combined action are small peptides and free amino acids, the small peptides accounting for more than 60% of intestinal amino acid nitrogen. The final digestion phase of most luminal peptides involves digestion by peptidases of the brush border and cytoplasm of intestinal mucosal cells, to produce free amino acids which are non-antigenic. These end-products of protein digestion are then transported across the mucosa via specific active transport systems and enter the portal blood flowing into the liver.

B. ABSORPTION AND DISTRIBUTION OF MACROMOLECULAR PROTEIN IN THE GASTROINTESTINAL TRACT

Along with amino acids and di- and tri-peptides, whole proteins and other large macromolecules are absorbed in amounts which are of little or no nutritional significance but may well be biologically or immunologically significant. Large molecules are absorbed intact by foetal and neonatal small intestine and several studies suggest that macromolecules can cross the mature mucosal barrier under normal physiological conditions, (Kilshaw and Slade, 1980: Warshaw *et al*, 1974: Swarbrick, 1979), and that there may be uptake of different amounts of macromolecules at different sites along the intestine

(Morris and Morris, 1976). One study in humans (Adibi and Mercer, 1973), demonstrated that protein can be found in both the jejunum and ileum as late as four hours after ingestion. These experiments illustrated the passage of protein right along the small intestine to the ileum, enabling the absorption of protein to occur all along the intestine.

One mechanism by which gut antigens may cross the mucosa, is by means of epithelial cell pinocytosis followed by the deposition of macromolecules in the interstitial space (Reviewed, Walker, 1981). Antigens can then enter the circulation if not taken up by macrophages, or by liver Kupffer cells. Some observations have also been made of intercellular absorption of macromolecules (Reviewed, Woloschak and Tomasi, 1983), and specialised antigen transport by M cells is one mechanism whereby antigens gain access to the gut lymphoid tissue (Walker, 1981).

Many different estimates of the amount of macromolecular protein crossing the mucosa into the systemic circulation have been reported by several workers using various experimental techniques. The results quoted range from greater than 40% of ingested dose being absorbed in rats (Morris and Morris, 1976), to as little as 0.01% of the ingested dose per millilitre of serum in mice (Hanson, personal communication, 1982; Strobel et al, 1983). Several experiments estimate antigen uptake from the gut into the systemic circulation by measuring levels of radiolabelled antigen in serum.(Hemmings et al, 1977; Seifert, et al, 197

Such experiments should be interpreted with caution since the presence of radioactivity may not be concomit ant with the presence of macromolecules unless appropriate precautions are taken to ensure that no free label appears in the plasma (Skogh, 1982), or that neither free label nor labelled protein

fragments are bound to endogenous plasma proteins (Udall et al, 1981). In experiments to be described in this thesis, immunological techniques have been used to assay macromolecular uptake from the gut. It should be borne in mind that even these techniques incur some overestimation since an antigen binding site detected by specific antibody may reflect antigen fragments bound to plasma proteins and not attached to a large molecule.

1.4 Immune Responses to Fed Antigens

The presence of antigen in the gastrointestinal tract may induce any of several different manifestations of local and/or systemic immune responses.

A. LOCAL IMMUNE RESPONSES TO ANTIGEN IN THE GASTROINTESTINAL TRACT

Immunoglobulins A, M, G and E are found in the secretions of the gut mucosa and it is now textbook knowledge that IgA is the predominant secretory antibody. A secretory antibody response can occur in the absence of serum antibody and so provide specific immunological resistance at mucosal surfaces. (Bienenstock and Befus, 1980, 1983; Heremans, 1974 ; Tomasi, 1976). However, not all mucosal responses are favourable for the host since Type-I hypersensitivity reactions (IgE-mediated), can occur in the gut causing oedema and reddening of the mucosa (Reviewed, Ferguson and Mowat, 1980).

The various T cell-mediated mucosal responses occurring as a result of antigenic stimulus in the gut are now well documented also.

Local delayed-type hypersensitivity has been described, (MacDonald and Ferguson, 1977; Mowat and Ferguson, 1981 a; Mowat and Ferguson, 1981 b), and cytotoxic T cells have been detected in Peyer's patches and extraintestinal sites after oral immunization (Kagnoff, 1978 a).

Other manifestations of local immunity in the gut include hyperplasia of mast cells and of goblet cells. Goblet cells release mucus in response to local antigen. In addition, Peyer's patch macrophages may provide non-specific resistance (MacDonald et al, 1982).

B. SYSTEMIC RESPONSES TO ANTIGEN IN THE GASTROINTESTINAL TRACT

The presence of antigen in the gastrointestinal tract can induce a variety of systemic immune responses ranging from humoral and cell-mediated immune responses, to suppression of either or both types of these responses, or even no net effect. The response induced is dependent on the dose and nature of antigen, the species, strain and age of experimental animal used and the type of antigen feeding protocol adopted in experiments.

For example, circulating antibodies have been observed after antigen feeding (Rothberg and Farr, 1965), and serum IgE can be induced by feeding small doses of antigen to rats given Bordetella pertussis systemically (Jarrett et al, 1976: Bazin and Platteau, 1976). Mice fed killed Escherichia coli showed enhanced serum antibody production in response to a subsequent parenteral immunization indicating that feeding the antigen had induced some degree of systemic priming (Stokes et al, 1979). This enhancement was associated with feeding small amounts of antigen whereas larger amounts of antigen or a more prolonged feeding regimen tended to induce hyporesponsiveness.

Systemic hyporesponsiveness of humoral and cell-mediated immunity following antigen feeding or, oral tolerance, is a long-established

phenomenon having been observed by Dakin in 1829 as a practice among Red Indians who ate Rhus leaves in order to prevent contact sensitivity, or "poison ivy" on later contact with the leaves. This phenomenon was later studied scientifically by Wells (Wells 1911; Wells and Osborne, 1911), and since then, oral tolerance has been studied in several species and strains of experimental animals and to various soluble and particulate antigens including contact sensitising agents (Chase 1946; Asherson et al, 1977), bovine serum albumin, in rats (Thomas and Parrott, 1974), sheep erythrocytes in mice (Kagnoff, 1978 b, c) and rats (Mattingly and Waksman, 1978), and ovalbumin in mice (Hanson et al, 1977; Hanson et al, 1979; Miller and Hanson, 1979; Richman et al, 1978; Ngan and Kind, 1978).

Serum antibody responses can be suppressed by feeding after systemic immunization (Lafont et al, 1982) and the responses of suckling animals can be suppressed by maternal ingestion of antigen (Jarrett and Hall, 1979; Peri and Rothberg, 1981). Orally induced systemic tolerance may also occur concurrently with an intestinal secretory antibody response (Challacombe and Tomasi, 1980; Stokes et al, 1979), and this may indicate a regulatory mechanism whereby antigen in the gut is dealt with locally, while inappropriate or even damaging systemic immune responses are suppressed.

1.5 Immune Regulation

A. REGULATION OF SYSTEMIC IMMUNE RESPONSES

Each response made by the immune system is highly amplified and subject to strict control. In the case of T lymphocytes, this control is exerted by products of the Major Histocompatibility gene complex or

MHC (Reviewed, Howie and MacBride, 1982).

The initiating event in an immune response is antigen recognition by lymphocyte surface receptors. These serve to bring cells of the immune system into contact with each other such that further signals may be transmitted between them depending on to which functional compartment the lymphocytes belong. The result of these signals may be activation, differentiation or suppression of target cells. Some lymphocytes recognize idiotypes, which are determinants associated with the antigen binding site of antibodies, and possess surface receptors for these determinants which are anti-idiotypic in nature and not anti-antigen. It is possible that interaction can also occur via anti-idiotype receptors on cells.(Reviewed, Urbain and Wuilmart, 1982). Examples of immunological signals are interleukins. These are molecules released by lymphocytes and antigen presenting cells which stimulate other cells of the immune system. Down-regulation of an immune response produces a state of immunological tolerance. This can be defined as a condition of specific hyporesponsiveness to an antigen which would be immunogenic in other circumstances. Lymphocytes of T or B classes, or both, can be tolerized in animals and, like a positive immune response, tolerance is influenced by the genetic constitution of the host, the mode of antigen presentation and the nature and amount of antigen presented. Tolerance mechanisms differ with experimental systems. In some experimental models, tolerance is associated with a loss of B cell activity (Aldo-Benson and Borel, 1974; Parks and Weigle, 1980 a), loss of helper T cell function (Parks and Weigle, 1980 b), induction of suppressor B cells (Asherson et al, 1977), and activation of suppressor T cells (Sy et al, 1977; Basten et al, 1974). In some instances, unresponsiveness can be

dissociated from T suppressor cell activity by criteria such as; kinetics of tolerance induction, drug sensitivity and dose of antigen required (Sherr et al, 1979; Colby and Strejan, 1980), and more than one regulatory mechanism may be active in the same animal.

B. SUPPRESSOR T LYMPHOCYTES

Suppressor T cells have often been implicated in the suppression of cell-mediated immune responses in mice (Sy et al, 1977; Asherson and Zembala, 1980), and different functional classes of suppressor cells, including suppressor memory cells, exist within this sub-population (Liew and Howard, 1980; Whisler and Stobo, 1978). Suppressor cells can act afferently, blocking the induction of responses, or efferently, preventing the action of effector cells after priming has occurred. Different types of suppressor T cells may be responsible at different stages of the cell-cell interaction pathway. For example, "auxiliary" suppressor cells are activated by antigen and can cause efferent suppressor cells to exert their inhibitory effect on T cells primed for a delayed hypersensitivity response (Sy et al, 1979). This illustrates the kind of complex pathways by which immune responses are regulated. Some of these complications could well disappear with future research since at present it is not clear whether some effector-suppressor cells represent mature activated forms of inducer-suppressor cells or are in fact separate sub-populations of cells (Germain and Benacerraf, 1981).

C. MUCOSAL IMMUNE REGULATION

The gut mucosa is in constant contact with various immunogenic and even potentially immunoregulatory substances. It is essential, therefore, that responses to antigens in the gastrointestinal tract are carefully regulated.

Oral tolerance, the systemic unresponsiveness observed following antigen feeding is a product of this regulation although the mechanism of this phenomenon is not clear and, like systemic immunity, may be governed by different mechanisms since serum antibody (Kagnoff, 1978 c), antigen-antibody complexes (André et al, 1975), and suppressor cells (Thomas and Parrot, 1974; Asherson et al, 1977; Ngan and Kind, 1978: Mattingly and Waksman, 1978), have all been reported.

T cell-mediated suppression following oral administration of antigen may extend only to IgM or IgG responses but not to secretory IgA (sIgA) responses since murine Peyer's patches after antigen feeding have been found to contain suppressor cells for IgM and IgG production together with helper T cells for sIgA responses (Richman et al, 1981). This is supported by the data of Challacombe and Tomasi (Challacombe and Tomasi, 1980), where antigen feeding induced systemic tolerance for antibody together with a salivary IgA response.

In vitro work using cloned T cells from Peyer's patches has revealed the presence of isotype-specific "switch" T cells which induce IgM-bearing B cells to differentiate into IgA-bearing B cells (Strober, 1982). These cells may be providing the net T cell "help" for IgA responses described in earlier work by the same group of workers (Richman et al, 1981).

Other in vitro studies have demonstrated cells of the contra-suppressor circuit in Peyer's patches. Contra-suppressor cells inhibit the activity of suppressor cells and their cell-free mediators and it has been postulated that contra-suppression allows immune responses to occur in microenvironments like the gut despite suppression of the systemic immune response (Green et al, 1982). There has, as yet been no in vivo demonstration of such a system

operating in the intestine.

Mucosal antibody, whilst being protective may also have a regulatory function in limiting the entry of antigen into the systemic circulation. Everted gut sacs from orally immunized rats show reduced passage of specific antigen across the mucosa when compared with gut sacs from non-immune rats (Walker and Isselbacher, 1972). This phenomenon is known as immune exclusion and although IgA can cause immune exclusion at the bronchial mucosal surface (Stokes et al, 1975), this function is not exclusive to IgA since both IgA and IgG were detected in washings and scrapings of the gut sacs used by Walker et al (1972, 1975). The presence of specific antibody at the gut mucosa of rats causes antigen to be absorbed on to the mucosal surface with the formation of immune complexes (Walker et al, 1975). This in turn stimulates mucus release from goblet cells allowing the rapid elimination of antigen to occur in the gut lumen (Walker, 1977). Immune exclusion is not just a property of everted gut sacs. Swarbrick measured the amount of antigen absorbed into the serum of mice after feeding and was able to demonstrate in vivo that oral immunization with a protein antigen reduces its subsequent absorption (Swarbrick, 1979).

Non-specific mechanisms such as gut flora, also feature in mucosal regulation. Bacterial lipopolysaccharide present in the gut lumen can induce suppression of immune responses (McGhee et al, 1980). On the other hand, prior activation of the reticuloendothelial system of mice leads to the abrogation of oral tolerance to ovalbumin and instead, results in an active mucosal delayed hypersensitivity response in the mucosa and mesenteric lymph nodes (Mowat and Parrott 1983). A regulatory role has also been suggested for the liver

Kupffer cells since re-routing of the gastrointestinal venous blood to by-pass the liver abrogates oral tolerance (Cantor and Dumont, 1967).

Appropriate regulation of mucosal and systemic immune responses is an important consequence of antigen feeding since perturbation of any of these regulatory mechanisms may evoke inappropriate and even harmful immune reactions either in the gut mucosa or systemically. This idea is borne out to some extent by data showing that the induction of a local CMI reaction in the intestinal mucosa following the abrogation of suppressor cell activity, led to changes in mucosal architecture similar to those seen in diseased intestines (Mowat and Ferguson, 1981). This gives some indication of the importance of active suppression of harmful immune responses to ingested antigens in protecting the host.

D. THE ROLE OF ANTIGEN STRUCTURE IN IMMUNE REGULATION

Several different determinants on complex antigens participate in the regulation of immune responses. This concept has arisen from experiments showing that antigens which have been structurally altered will evoke quite different sorts of immune responses compared to the original antigen and even influence subsequent responses to that original unaltered antigen. Deaggregated protein antigens are tolerogenic (Dresser, 1962) for example, and fragments of bacterial flagella antigens obtained by Cyanogen Bromide cleavage suppress responses to the intact antigen (Ada and Parish, 1968). It is also well known that heat-aggregated protein antigens will elicit a more powerful delayed hypersensitivity (DTH) reaction in mice primed with native antigen than the native antigen itself (Titus and Chiller, 1981). An increase in antigenicity proportional to the loss of

α-helix content in protein tertiary structure was achieved by mercaptoethanol reduction of BSA. As the helix conformation is lost, it is possible that either new antigenic determinants are created or that hidden determinants are revealed thereby increasing the antigenic stimuli associated with the protein (Goetzl and Peters, 1972).

In early studies on DTH, heat denaturation of soluble protein antigens altered the antigenicity of the protein with respect to antibody production whilst DTH responses were identical to either native or heat denatured protein (Gell and Benacerraf, 1959). This split in responsiveness of the humoral and cell-mediated arms of immunity due to altered antigen structure reflected the different structural requirements for the induction of B and T cell responses against complex protein antigens. Further experimental support for this idea was provided when urea-denatured antigens were found to be capable of affecting T cells specific for the native antigen and causing suppression of the serum IgE response to the native antigen despite the destruction of the majority of serologically detectable determinants during denaturation (Ishizaka et al, 1979). The suppression of IgE responses by modified antigen has potential as a therapy for allergic disease and indeed, in animal models, allergens and allergenic haptens can be converted to non-immunogenic and tolerogenic derivatives by conjugation to non-immunogenic synthetic polymers (Reviewed, Sehon, 1982).

Antigen fragments which are not recognised by B cells by virtue of having no antibody binding sites can suppress antibody responses by activating suppressor T cells (Michael et al, 1981), and the different T cell subclasses may also possess different structural requirements for their activation. Suppressor T cells induced in response to

urea-denatured ovalbumin (OVA) suppress the *in vitro* proliferative response to urea-denatured OVA and do not suppress the response to native OVA. In a likewise manner, suppressor cells induced to native OVA do not suppress the response to urea-denatured OVA. However, helper T cells in the same experimental system will recognise both native and denatured antigen regardless of which antigen form they are induced with (Endres and Grey, 1980 a). These results seem at first to conflict with the data of Ishizaka *et al*, (1979), however, both groups used slightly different methods to prepare the urea-denatured antigen and measured suppression in different arms of the immune response; in one case the IgE response and in the other the proliferative response. These types of responses could well involve different pathways of cell interactions or even different suppressor cell populations.

Genetically determined high and low responder mice have proved useful in investigating the importance of antigen structure in immune responses under Ir gene control. For example, H-2^b and H-2^s mice are low antibody responders to hen egg white lysozyme because they produce a predominance of suppressor cells specific for a suppressor determinant on the molecule which is 27 amino acids long and known as the N-C peptide (Sercarz *et al*, 1978; Adorini *et al*, 1979). In elegant experiments where the N-C peptide was excised, the low responder mice behaved as high responders and produced helper T cells specific for a different portion of the molecule; the so-called LII fragment. This illustrates that suppressor and helper T cells respond to different structural determinants on the same molecule and that strain differences in responsiveness may reflect immunodominance of either helper or suppressor determinants. In these experiments it is

interesting to note that the suppressor determinant is a peptide of only 27 amino acids. Different T cell subclasses then, are capable of recognizing fragments of globular protein antigens, in this case fragments perhaps conserving only part of the primary protein sequence, and can affect the response to the whole native protein. That T cells recognise primary protein structure has previously been proposed by Benacerraf, (1978), in reference to "processed" antigen associated with Ia molecules on the surface of antigen presenting cells.

Antigen processing and presentation in vivo by macrophages and other antigen presenting cells allows for the selection and presentation of the appropriate antigenic determinants to lymphocytes and there is some indirect evidence that antigen which is not processed by macrophages preferentially activates suppressor T cells (Endres and Grey, 1980 a; Yoshikai et al, 1981). In addition, animal strain differences in susceptibility to tolerance can reflect differences in antigen handling by macrophages either in processing or presentation of antigen in the induction phase of immunity. For example, in one study C57Bl/6 mice were more easily tolerised by deaggregated human γ-globulin (HGG) than were BALB/c J mice. This was due to inefficient processing by C57Bl/6 macrophages of trace amounts of HGG aggregates in the deaggregated antigen preparation (Golub and Weigle, 1967). Further to this, BALB/c mice given Carageenan which poisons macrophages, lost their "resistance" to tolerance induction with deaggregated protein, (Lukic et al, 1975). Clearly the nature of antigen presented in the immune system can exert a powerful regulatory influence.

E. CONSIDERATION OF ANTIGENIC STRUCTURE AND MUCOSAL IMMUNE REGULATION

By virtue of the digestive and absorptive functions of the gut, protein antigen entering the host via the gastrointestinal tract is more likely to experience structural alteration than native protein injected systemically. Antigen structure is a determining factor in the outcome of immune recognition and so the gut feasibly has a unique role to play in influencing antigen conformation which will in turn influence the type of immune response evoked by feeding antigen.

Studies on cholera antigen have shown that different antigen forms presented at mucosal surfaces will produce changes in the kinetics and magnitude of the mucosal antibody response (Pierce, 1978). However, little is known of the antigen forms presented to the systemic immune system via the gut which occur as products of the combined physiological and immunological properties of the intestine. These include digestion, immune exclusion, absorption, and can extend to include liver filtration. This combined effect can be described as gut-processing of antigen or antigen handling by the gut and is dependent therefore, on the genetic and immunological constitution of the host (Swarbrick, 1979), the physiological status of the gut and the molecular conformation of the antigen. Ovalbumin for example, has a very rigid structure and there is some evidence that this protein is not readily digestible with trypsin or chymotrypsin *in vitro* (Yokota *et al*, 1982).

In one *in vivo* study of antigen handling by the gut, serum was collected after feeding ovalbumin to mice and assayed by gel filtration. Ovalbumin was found only in the fraction corresponding to the molecular weight of native ovalbumin (Swarbrick, 1979). This is

at variance with the implications of the *in vitro* experiments of Michael *et al*, who found that fragments of bovine serum albumin obtained by *in vitro* proteolysis with pepsin suppress the antibody response to native bovine serum albumin when injected into mice prior to immunization. These fragments induce suppressor T cells in the mice and will tolerize B cells if conjugated to homologous γ-globulin (Michael *et al*, 1981). Whether similar fragments are generated in the circulation after *in vivo* protein digestion is not clear at present and was a subject for study in this thesis.

1.6 Approaches to the Study of Immune Regulation in the Context of Gastrointestinal Processing of Antigen

In order to investigate the roles of both antigen handling by the gut and suppressor T cells in oral tolerance, and to learn more of the relationship between mucosal damage and immunity, systemic immune responses were studied in animals which had been compromised by disruption either of their normal regulatory mechanisms of immunity or of their normal gut functions. This disruption was achieved by treating mice with the drug cyclophosphamide: (this had been extensively studied in the research programmes of Dr Allan Mowat and Dr Stephan Strobel in this laboratory, investigating the effect of the drug on oral tolerance control mechanisms), and also by subjecting mice to whole body irradiation.

A. CYCLOPHOSPHAMIDE

Cyclophosphamide (CY), is an alkylating agent which, upon injection into the host, is rendered biologically active by the liver and can then disrupt DNA synthesis in host cells, (Foley *et al*, 1961). It follows that a primary target for this drug will be actively

dividing cells and so when CY is injected shortly after immunization of the host, when cells of the immune system are normally in a state of proliferation, cell division is inhibited and the immune response is suppressed (Ramshaw et al, 1976). If injected prior to immunization, CY can either selectively enhance or deplete cell-mediated immunity depending on the dose of CY administered. For example, a dose in the 20-200 mg per kilogram body weight range enhances delayed hypersensitivity responses (Diamantstein et al, 1981; Atallah et al, 1979). In some cases, increased cell-mediated immunity due to CY treatment is associated with depletion of B cells from lymphoid tissue (Turk and Parker, 1982). CY will reverse tolerance when suppressor cells are responsible (Turk and Parker, 1982; Sy et al, 1977), and can also enhance immune responses following their depression by antigenic competition (Dwyer et al, 1981).

Cells of the intestinal epithelium are constantly in a state of renewal (Reviewed, Leblond, 1980), and as such are sensitive to the alkylating properties of CY. Cyclophosphamide administration causes rapid inhibition of crypt cell mitosis followed by a regeneration period during which time crypt cell proliferation is higher than normal and crypt cell death and extrusion can be seen (Rosenoff et al, 1975; Ecknauer, 1976; Young 1976; Sobhon et al, 1977). It is not known whether these morphological changes reflect any difference in the absorptive capacity of the gut or of antigen handling, and in this respect the dose of CY may be important since a single dose of 100 mg/kg of CY which is sufficient to produce morphological changes, only minimally affects enzyme activity or sugar absorption in the gut epithelium of rats (Ecknauer, 1976).

B. IONIZING RADIATION

Organised lymphoid tissues and recirculating small lymphocytes are exquisitely radiosensitive and the effects of radiation on all aspects of immunity are now well documented (Taliaferro et al, 1964; Anderson and Warner, 1975). Briefly, the death of cells due to irradiation can be defined as either (i) reproductive death where the ability of the cell to divide is destroyed, or (ii) interphase death which is characteristic of small lymphocytes and describes the death of cells almost immediately after exposure to irradiation without mitosis occurring. Small B cells are more sensitive than small T cells (Anderson et al, 1974), and activated T cells are resistant to radiation-induced interphase death as they have developed beyond the proliferation stages of differentiation and clonal expansion (Hamaoka et al, 1972).

One important source of disruption of immune mechanisms following irradiation is its effect on cell traffic (Reviewed, Anderson and Warner, 1975). Whole body irradiation causes a sharp decrease in numbers of recirculating small lymphocytes due to the impaired capacity of these cells to traffic normally. This dysfunction was illustrated by Kettman and Matthews who showed that delayed hypersensitivity to sheep erythrocytes can be transferred into normal mice only when the irradiated primed spleen cells are injected with antigen directly into the footpad. An intravenous injection of these cells fails to sensitise normal mice because normal cell traffic is impaired (Kettman and Matthews, 1976).

In the intestine, the constant division and renewal of cells in the intestinal epithelium renders this organ highly sensitive to radiation damage (Reviewed, Quastler, 1962). Within one hour following irradiation, focal oedema of villi can be detected along

with distortion of crypt cell proliferation, mitosis being virtually abolished by 24 hours. In addition, cells are not extruded from the tips of the villi for the first 12 hours following irradiation. In rats, shorter villi and longer crypts are seen within a few days due to regeneration of epithelial cells over the damaged mucosa. The villous epithelium is recovered in appearance after 5-7 days although intestinal function, which is lowest 3 days after irradiation begins to recover at 4-6 days, with full recovery taking as long as 10-17 days (Detrick et al, 1963).

1.7 Ovalbumin

The protein ovalbumin (OVA) was chosen as an antigen for use throughout the work of this thesis for a variety of reasons: it is a soluble protein, available in commercially purified form and is not normally encountered in the diet of mice reared in this animal unit.

Various physical and chemical properties of OVA have been comprehensively reviewed (Vadehra and Nath, 1973): ovalbumin constitutes around 54% of the protein in egg white and is usually isolated by crystallisation. It has a molecular weight of 43,500 daltons and is the only egg white protein which bears free sulphydryl groups. Ovalbumin also has a carbohydrate moiety which comprises 3.5% of the molecule. The carbohydrate is mainly in the form of mannose with a small amount of galactose also present. The dominant amino sugar of OVA is glucosamine but fucose and sialic acid residues are also present in minute quantities. Ovalbumin is easily denatured by urea, guanidium salts, thiocyanate and heat, in which case aggregates of varying length are produced depending on the concentration of OVA and time of heating. Other physical parameters which change during

denaturation include viscosity, light scattering and ultracentrifugal behaviour with minimal changes observed on electrophoresis of native and denatured OVA.

The sequencing of messenger RNA for chicken ovalbumin has revealed information about the primary amino acid sequence of OVA. This consists of 385 amino acid residues from N-terminal acetyl-glycine to C-terminal proline with carbohydrate attached to the molecule at Asparagine-292. Ovalbumin is also a phosphorylated protein and has a single intra-chain disulphide bond. In addition, different genetic variants or allelic forms of OVA exist (McReynolds et al, 1978).

Immunochemical analysis of crystalline OVA shows the presence of 3 fractions: A1, A2 and A3, which differ from each other on the basis of phosphorous content. A3 is the molecule initially synthesised and is most susceptible to denaturation. A2 and A1 are transformed varieties of the original protein with A2 being more susceptible to denaturation than A1 (Rhodes et al, 1958: Vadehra and Nath, 1973). Ovalbumin has been identified as a major allergen in hen's egg white (Langeland, 1982), and part of its antigenic determinants have been located in the amino acid sequence from leucine 105-lysine 189 (Yokota et al, 1982).

CHAPTER 2

MATERIALS AND METHODS

SECTION (a)

GENERAL MATERIALS AND METHODS

Animals

BALB/c mice and BDF1 mice (crosses between DBA/2 males and C57B1/6J females) were bred and maintained in the Animal Unit of the Western General Hospital, Edinburgh. Adult male and female animals were used mainly at between 6 and 8 weeks of age. They were fed *ad libitum* on normal laboratory rodent diets which contained no chicken or egg proteins (Spratt's No. 1; Spratt's Patent Ltd: CRM (X); Labsure) and allowed unlimited access to tap water. Unless otherwise stated, groups of 5 to 6 animals were routinely used for experiments.

Antigens

Ovalbumin or OVA (chicken albumin, Grade V; Sigma Chemical Co.) was most frequently used throughout the work. Human serum albumin or HSA (Sigma) was used as a non-cross-reacting protein.

Denatured OVA was prepared according to the method of Chesnut, Endres and Grey (1980): OVA (15mg/ml) was dissolved in 0.1M Tris buffer (BDH) at pH 8.2. Solid Urea (Sigma) was added to a final concentration of 8M and 2-mercaptoethanol (Sigma) to a final concentration of 0.2M.

Reduction of native OVA was allowed to proceed for 12 hours at room temperature, then, exposed sulphydryl groups on the protein were alkylated by adding 0.3M iodoacetate (Sigma). This prevented the spontaneous re-folding of the molecule. The mixture was left stirring at room temperature for 2 hours during which time pH7 was maintained

by the addition of NaOH. The protein was then dialysed against large volumes (about 2 litres) of 0.05M Na_2 HPO_4 and then against phosphate-buffered saline, PBS. The resultant solution was filter sterilised using a 0.2μm millipore filter and stored at 4°C.

PBS was made up according to the following formula:

Annhydrous	Na_2HPO_4	1.50g
Annhydrous	K_2PO_4	0.43g
	NaCl	7.2g

dissolved in distilled water to a final volume of 1 litre.

pH 7.2

Deaggregated OVA was prepared according to the method of Colby and Strejan (1980): a solution of OVA in distilled water (5 mg/ml) was centrifuged for 3 hours at 100,000 g in a Beckman Spinco (Model L) ultracentrifuge. the top one-third of the solution was then collected and injected into the mice on the same day, as deaggregated OVA tends to spontaneously re-aggregate.

Anaesthesia

All procedures with BDF1 mice were carried out using light ether anaesthesia. BALB/c mice were anaesthetised for footpad injections and blood collections.

Injections

Intra-venous (i.v.) injections in mice were made via the retro-orbital venous plexus using a 26 gauge needle.

Intra-peritoneal (i.p.) injections were carried out using a 25 gauge needle.

Immunization Procedure

Mice were immunized with 100 µg antigen emulsified in Freund's complete adjuvant or FCA (H37Ra, Difco) and injected intradermally into one rear footpad (injection volume, 0.05 ml) using a 26 gauge needle.

Feeding Procedure

Mice were fed with antigen or control solutions by intragastric intubation using a 19 gauge stainless steel dosing needle with a rounded tip. For oral tolerance induction, mice were fed without previous fasting, with 25 mg OVA dissolved in 0.2 ml of either sterile water or sterile physiological saline (0.15M NaOH).

Blood Collection

Up to 200 µl of blood were obtained routinely from the retro-orbital plexus under ether anaesthesia using heparinised haematocrit tubes (Propper Ltd, Long Island, New York). This route was chosen for survival experiments. Larger quantities of blood were obtained by either cardiac puncture or bleeding mice from the axillary vessels. Haematocrit tubes were sealed with CRISTASEAL (Hawksley, England) and allowed to clot in the upright position before being spun in a haematocrit centrifuge (Hawksley, England) for five minutes. Sera obtained for testing in haemagglutination assays were decomplemented for 30 minutes in a water bath at 56°C. All sera were stored at -20°C.

Weighing Mice

Mice were weighed on an Oertling TD30 top pan balance.

Cyclophosphamide Treatment

Mice were weighed and then injected i.p. with 100 mg per kg body weight cyclophosphamide or CY (Endoxana) dissolved in sterile water.

Irradiation of Mice

Mice were placed inside a perpex jig. They were irradiated at a rate of 38 rads per minute and received a total dose of 1,000 rads from a 250 megavolt orthovoltage x-ray source (Siemens Orthovolt).

This procedure was very kindly carried out by Mrs Barbara Clarke and Miss Sandra Biggar, Radiation Oncology Unit, The Western General Hospital, Edinburgh.

Spleen Cell Reconstitution

Spleens were removed from BDF1 mice which had been killed by cervical dislocation. A suspension of spleen cells in RPM1 1640 medium (Flow Laboratories Ltd), was prepared by cutting the spleens into tiny pieces with fine scissors and then forcing the tissue through a fine mesh metal strainer using the plunger of a 5 ml disposable syringe. The suspension was sucked up and down in a syringe to disperse any clumps before the cells were washed and counted. Cells were counted in an improved Neubauer haemacytometer slide chamber using Trypan Blue Dye exclusion as a means of identifying viable cells from the total number of cells which was estimated using white cell diluting fluid. The concentration of viable cells was adjusted to 5×10^8 cells per ml and recipient animals were injected i.v. with 0.05 ml (25×10^6 cells) of the suspension.

Assessment of Systemic Delayed-Type Hypersensitivity

Mice were tested for delayed-type hypersensitivity (DTH) with microcalipers (POCOTEST-A, Carobronze Limited, London, United Kingdom)

by measuring the increment in footpad thickness 24 hours after an intradermal injection of 100 µg of antigen in 0.05 ml of saline into the plantar side of a non-immunised rear footpad. Control mice were challenged with saline. The increment in footpad thickness in response to saline was almost always zero or less than zero.

Passive Haemagglutination Assay

In some experiments, sera were tested for antibodies by passive haemagglutination. Sheep red blood cells were collected and stored in Alsever's solution* and washed three times in saline at 150 x g before use. 100 µl packed sheep red blood cells were mixed with 0.7 ml saline and 100 µl protein solution (15 mg/ml ovalbumin) in saline. 1 ml of 0.01% chromic chloride (Analar, BDH Limited) in saline at pH 5.0 was added dropwise under continuous agitation. The mixture was then allowed to stand for 10 minutes at room temperature (around 25° C). The reaction was stopped by adding 10 ml of PBS and the coated cells were then washed twice in PBS and resuspended at 1% before use.

Decomplemented sera were absorbed with 10% sheep red blood cells for about one hour before use. 25 µl serum was doubly diluted with physiological saline in round bottom microtitre plates (TITERTEK, Flow Laboratories) and 25 µl of coated sheep red blood cells were added with a multi channel (eight) pipette (TITERTEK, Flow Laboratories) to each well.

After settling for 90 minutes at room temperature, the titres were taken as the last dilution to show complete agglutination. All sera were tested with or without the addition of 25 µl of 0.1 M 2-mercaptoethanol (Sigma Chemical Co Ltd) to obtain information on both mercaptoethanol-resistant and sensitive antibodies.

* Alsever's Solution

dextrose	20.5 g
Na Cl	4.2 g
sodium citrate	8.0 g

sterilised by membrane filtration (0.2 μm)

stored at room temperature.

Detection of Anti-ovalbumin Antibodies in Mouse Serum Using An Enzyme-Linked Immuno-Sorbent Assay (ELISA).

(i) ELISA REAGENTS

Carbonate buffer; 0.05 mol/litre at pH 9.6 (Northeast Biomedicals Ltd).

Washing solution; 0.05% Tween 20 (BDH) in physiological saline.

Serum Diluent; 0.05% Tween 20 in physiological saline with 0.2% sodium azide (BDH).

Substrate buffer; 100 ml DEA (diethanolamine; BDH)

800 ml H_2O

0.1015 g Mg Cl_2. 6H20 (BDH)

0.2 g sodium azide

pH 8.6

(ii) ELISA FOR ANTI-OVA ANTIBODIES

This assay was developed by Dr Stephan Strobel and Mrs Margaret Gordon. Mrs Gordon also carried out these assays in my experiments. OVA was dissolved in carbonate buffer at a concentration of 1 mg/ml and 125 μl of this solution was added to each well of a microtitre plate (Linbro; Flow Laboratories Ltd) and incubated for 16 hours

(overnight) at 4°C. All incubations were carried out in a moist box to prevent "edge effects". The wells were then washed 3 times with ELISA washing solution.

Mouse sera to be tested, including hyperimmune mouse reference serum as a positive control, were diluted 1 in 100 in serum diluent and added in 125 µl volumes to the wells. The plate was incubated at room temperature for 5 hours and then washed 3 times with ELISA washing solution.

A dilution of 1 in 1750 in serum diluent of rabbit anti-mouse IgG conjugated to alkaline phosphatase (Miles-Yeda Ltd) was added to each well in 125 µl volumes. The plate was incubated for 16 hours at room temperature and then washed 3 times as before.

Substrate solution was added (1 mg/ml p-nitrophenyl phosphate; Sigma 104 phosphatase substrate tablets in substrate buffer), 125 µl per well, and incubated for approximately 30 minutes at room temperature. The plate was then read using an automatic ELISA reader (Dynatech Ltd) set at wavelength 405 nm.

Detection of anti-HSA Antibodies in Mouse Serum by ELISA

HSA was dissolved, 15 mg/ml in carbonate buffer and 125 µl of this solution was added to each well of a microtitre plate. The plate was incubated for 16 hours (overnight) at 4°C and then washed 3 times with ELISA washing solution.

Reference and test sera were diluted 1 in 100 in serum diluent and added in 125 µl volumes to the wells. The plate was incubated at room temperature for 2 hours and then washed 3 times with ELISA washing solution.

A dilution of 1 in 1750 in serum diluent was made of the rabbit anti-mouse conjugate (used for the anti-OVA ELISA) and 125 µl added to

each well. This was followed by a 3 hour incubation at room temperature.

The plate was washed 3 times with ELISA washing solution and then 125 µl substrate solution was added to each well. After an incubation of approximately 35 minutes at room temperature, the plate was read at wavelength 405 nm.

This assay was developed and performed by Mrs Margaret Gordon.

Detection of OVA in Mouse Serum by ELISA

Acrylic microtitre plates (Falcon; Becton and Dickinson) were coated with 150 µl of specifically-purified IgG anti-ovalbumin antibody (the generous gift of Dr Donald G. Hanson) diluted in carbonate buffer (10 µg/ml) for 2 hours at 37° C. After this incubation, plates were washed 3 times with ELISA washing solution.

Standard concentrations of OVA were obtained by making logwise dilutions of OVA in normal mouse serum and were diluted a further 1 in 10 in serum diluent before being added to wells in 125 µl volumes.

Plates were incubated for 16 hours (overnight) at room temperature and then washed 3 times with ELISA washing solution. Conjugate (Rabbit IgG-anti-OVA coupled to alkaline phosphatase; Northeast Biomedicals) was diluted 1 in 500 in serum diluent and 125 µl added to each well. Plates were incubated at room temperature for 24 hours and then washed 3 times with ELISA washing solution. Substrate solution was then added, 125 µl per well, and the plates were read at wavelength 405 nm.

This assay was developed by Mrs Barbara Walton and performed in my experiments by Mrs Hazel Drummond.

Collection of Samples for Histology

Mice were killed by cervical dislocation. Immediately afterward, pieces of jejunum about 5 mm long were removed at 10 cm from the pylorus, placed on a piece of card and cut open. The cards were placed in about 10 ml of fixative.

Fixing and Staining Histological Specimens

Samples for conventional histology were fixed in 10% buffered formalin and embedded in paraffin wax. Sections 5 microns thick were cut and stained with haematoxylin and eosin.

Histological processing was carried out by Mr Alexander Sutherland.

Intraepithelial Lymphocyte Counts

Intraepithelial lymphocyte counts are expressed as the number of intraepithelial lymphocytes/100 villus epithelial cells. Sections were examined under x 1000 (oil immersion) magnification and only well cut sections with a single epithelial cell layer were counted.

Counts were done by enumerating epithelial and lymphoid cell nuclei lying unequivocally above the basement membrane and a total of 500 cells were counted in each specimen. In serially sectioned specimens care was taken to avoid recurring areas.

Measurement of Intestinal Villi and Crypts

Pieces of jejunum were fixed in Clarke's fixative for maximum 24 hours. Thereafter they were transferred to 75% ethanol for storage before microdissection. To allow repeated examination in case of technical difficulties, only half of the tissues were stained in bulk

by the modified Feulgen reaction. Pieces of gut were immersed in 50% ethanol for 10 minutes, followed by tap water for 10 minutes and 7 minutes hydrolysis in 0.1 N HCl at 60°C. The tissue was then rinsed 3 times with tap water and stained with Schiff reagent (Difco Limited) for 20-30 minutes at room temperature and then kept in tap water (maximum 48 hours) for microdissection.

The lamina muscularis mucosa was removed under the dissecting microscope (x32, Zeiss Stereomicroscope 4B) and a single villus or thin segments of mucosa containing a few villi and their crypts were then cut out of the non-traumatised mucosa by dissection with a cataract knife (Weiss Limited). The mucosal fragments were placed on a slide in a drop of 45% acetic acid, covered with a coverslip and examined under a microscope with a previously calibrated eyepiece micrometer.

In each specimen, the lengths of 10-15 complete villi and crypts were measured and the means taken for group comparisons.

Counts of intraepithelial lymphocytes and measurements of intestinal villi and crypts were performed in collaboration with Dr Stephan Strobel.

Fixatives Used in Histology

(i) 10% buffered formalin (1 litre):

Formalin (40% formaldehyde)	100.0 ml
H_2O	900.0 ml
$Na_2HPO_4.2H_2O$	4.0 g
Na_2HPO_4	6.5 g

(ii) Clarke's fixative (1 litre)

ethanol (96%)	750.0 ml
glacial acetic acid	250.0 ml

Statistical Analysis and Presentation of Data

In general, results are presented as means +1 standard error of the mean (s.e.) or +1 standard deviation (s.d.) as stated in figures and tables.

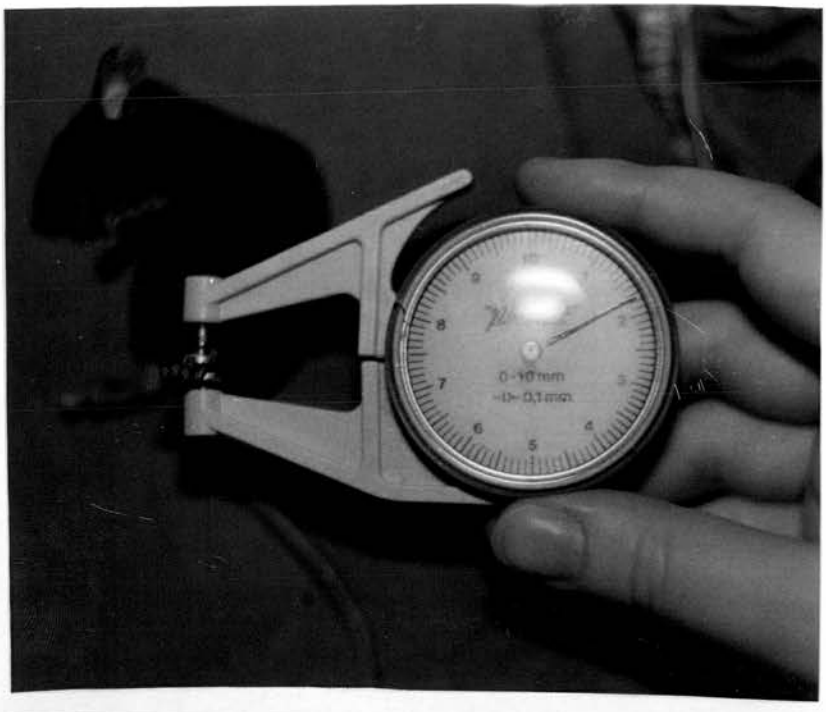
Students t-test was used to compare differences between group means in most cases. Where between-group comparison by t-test was innappropriate has been indicated in tables by a dash (-).

Antibody levels measured by ELISA are expressed as units of optical density at 405 nm and were compared by Wilcoxon Rank Sum tests since non-parametric distribution of this data could not be ruled out.

P values of greater than 0.05 are indicated as N.S. (not significant).

Results which were below the levels of detection of an assay are described either as "below background" as in the case of lymphocyte transformation tests, or "negligible" (neg.) as in the ELISA to detect OVA in serum.

Calculations were done using either a Sharp EL-58A or a Casio fx-3600P calculator.



A

B

Plates A and B

DTH response to OVA elicited in the footpad 21 days after OVA immunization.

A : pre-DTH challenge, footpad thickness = 1.8mm

B : 24 hours after DTH challenge, footpad thickness = 1.9mm

C

Plate C

DTH response to OVA elicited in the footpad 21 days after OVA immunization.

Upper footpad : 24 hours after saline challenge

Lower footpad : 24 hours after OVA challenge

SECTION (b)

DEVELOPMENT OF AN ANTIGEN-DRIVEN MOUSE T CELL TRANSFORMATION ASSAY

Introduction

This assay was developed based on the *in vitro* lymphocyte migration inhibition assay of Mowat and Ferguson, (1981)a, and the mouse T lymphocyte transformation assays of Corradin *et al* (1979), and of Kagan and Ben-Sassoon (1980). There were 3 basic stages in the development of this assay:

(i) Determination of the concentration of radio-labelled Thymidine, cell number and mitogen dose for non-specific transformation;

(ii) Determination of the optimum cell number and antigen dose for OVA-specific transformation;

(iii) Confirmation of the T cell-dependency of OVA-specific transformation.

In this section I shall first of all describe the assay and then the experiments leading to its development.

Mouse T Lymphocyte Transformation Assay Medium

The culture medium consisted of RPMI 1640 medium (Flow Laboratories Ltd) supplemented with 2 m.mol/l glutamine (Flow Laboratories Ltd), 100 I.U./ml Penicillin and 100 µg/ml Streptomycin (Flow Laboratories Ltd), 50 µ.mol/l 2-mercaptoethanol (Sigma), 5% human AB+ serum (a generous donation from Dr B MacLelland, Edinburgh

and S.E. Scotland Regional Blood Transfusion Service) and buffered with 10 m mol/l HEPES buffer at pH 7.2 (Flow Laboratories Ltd).

Antigen and Mitogen

Ovalbumin or OVA (egg albumin, Grade V, Sigma) was used at a concentration of 1.25 mg/ml, that is, 250 µg per 200 µl culture well and the T cell mitogen Phytohaemagglutinin or PHA (Wellcome) used at a concentration of 0.45 mg/ml in cultures.

Preparation of a Lymphocyte Suspension

Mice were killed by cervical dislocation and pinned out on a cork dissecting board which had been swabbed with alcohol. Mice were also swabbed before dissection to prevent loose fur from contaminating the cell preparation. Whenever possible, separate sets of sterile instruments were used for cutting open the skin and for removing the lymph nodes. From mice which were consistently immunized in the right hind footpad, were removed the right popliteal and inguinal lymph nodes and when necessary, the mesenteric lymph nodes. These were trimmed of excess fat before being placed in a sterile petri dish containing a little medium.

Lymph nodes were teased with fine scissors and then forced through a fine mesh metal strainer using a 5 ml disposable syringe plunger. The resultant cell suspension was sucked up and down in a syringe, in order to disperse any clumps, and then transferred to a sterile universal bottle which was topped up with fresh medium.

The suspension was washed 3 times in fresh medium by centrifugation at 400 g for 7 - 10 minutes per wash in either an MSE Super Minor or MSE Multex centrifuge. After the final wash, the

number of viable cells in the suspension was estimated by subtracting the number of cells staining with Trypan Blue dye from the total number of lymphocytes, stained with white cell diluting fluid. Cells were counted on an Improved Neubauer haemacytometer slide using a Leitz Ortholux microscope. The concentration of viable cells was adjusted to 2.5×10^6 cells/ml in culture medium containing antigen or mitogen where appropriate.

Treatment of Cells with Anti-Thy 1.2 and Complement

A suspension of lymph node cells were prepared and counted. Cells were suspended at a concentration of 10×10^6 cells per ml in 5 ml anti-Thy 1.2 (F7DF monoclonal IgM, cytotoxic antibody; OLAC 1976 Ltd) diluted 1 in 1 000 in RPMI 1640 and left for 30 minutes at room temperature. After this incubation, 5 ml complement (fresh guinea pig serum diluted 1 in 5 in sterile saline) was added to the suspension and this was incubated a further 40 minutes at 37 °C.

The cells were then re-counted, washed once and re-suspended in fresh culture medium at a concentration of 2.5×10^6 cells per ml. Control samples included untreated cells and cells treated with complement only. The controls were subjected to the same incubation times and temperatures as the test samples.

Culturing Cells

Cultures were set up according to experimental protocol, in quadruplicate wells containing a total volume of 200 µl each in sterile, 96-well, flat-bottomed Microtiter plates of tissue culture grade with lids (Flow Laboratories Ltd). The culture plates were handled and opened only inside a Microflow cabinet (Microflow Ltd)

with uni-directional air flow. Cultures were incubated in a Hotpack humidified CO_2 incubator at 37°C with 80% humidity in a mixture of 5% CO_2 and 95% air. The day of setting up a culture was called day 0.

Radio-Labelling Cultures

On day 4 of culture, the cells were pulsed with 0.01 µCi per well of C^{14} -labelled Thymidine (Amersham International) diluted in sterile saline. Some wells were always left unlabelled as controls for machine background radiation counts.

Harvesting Cultures

On day 5, at 18-20 hours after pulsing, the cells were harvested onto Titertek filter paper discs (Flow Laboratories Ltd) using an automated cell harvester (Dynatech Automash). The discs were allowed to dry thoroughly and were then placed in plastic minivials (Scintillation mini vials, A & J Beveridge Ltd) inside glass scintillation vials (A & J Beveridge Ltd). These were filled with 2.5 ml Koch-lite scintillation liquid (A & J Beveridge Ltd) inside a fume-hood and sealed.

C^{14}- Thymidine uptake was measured as counts per minute (cpm) using a Packard Tri-Carb Liquid Scintillation Spectrometer which measured β emission. The counting efficiency of the machine was checked by comparing the cpm obtained from a filter disc labelled with a standard 0.01 µCi with the formula:

I µCi = 2.2×10^6 disintegrations per minute. During the period of time when these assays were being performed, the counting efficiency of the machine was 50%.

Data Handling

The mean background cpm was calculated and subtracted from all counts before the mean cpm of quadruplicate wells was calculated. A stimulation index (SI) was calculated where appropriate from the formula:

$$SI = \frac{\text{mean cpm of stimulated wells}}{\text{mean cpm of unstimulated wells}}$$

Determination of C^{14}-Thymidine Concentration, Optimum Cell Number and Mitogen Dose For Non-Specific Transformation

Test cultures were set up using lymph node cells from naive BDFI mice. In one culture, an arbitrary cell concentration of 2×10^5 cells per well was chosen and the cells were either left unstimulatd or were stimulated with 0.45 mg/ml PHA. The cells were labelled on day 4 with either 0.1 μCi or 0.01 μCi C^{14}-Thymidine.

From the results of these tests (Table (a)) it was found that as little as 0.01 μCi C^{14}-Thymidine added to PHA-stimulated cells could produce high counts against a very low background of cpm in labelled unstimulated cells. This concentration of isotope was then adopted for use in every subsequent assay.

In a second culture, the response of various numbers of cells to PHA was tested including the response to PHA employed at the 5 times higher dose of 2.25 mg/ml. The optimum response was seen when the lower dose of PHA, 0.45 mg/ml, was used and for all cell concentrations, with a slightly better response occurring in the range 3×10^5 - 5×10^5 cells per well (Table (b)).

Added to 2 x 10^5 cells	Mean cpm ± s.e.
0.45 mg/ml PHA, 0.1 μCi C^{14}	11,140 ± 3,054
no PHA 0.1 μCi C^{14}	26 ± 18
0.45 mg/ml PHA, 0.01 μCi C^{14}	5,400 ± 3,015
no PHA 0.01 μCi C^{14}	5 ± 6

TABLE (a)

PHA-Response of Naive Lymph Node Cells Detected By Two Doses of C^{14}-Thymidine

Cells were obtained from BDFI mice. Cultures were labelled with either 0.1 μCi or 0.01 μCi C^{14}-Thymidine.

Number of cells per well	mg/ml PHA	Mean cpm ± s.e.
6×10^5 cells	2.25	696 ± 418
5×10^5 cells	2.25	2,795 ± 756
4×10^5 cells	2.25	2,363 ± 270
3×10^5 cells	2.25	880 ± 115
6×10^5 cells	0.45	4,243 ± 1,054
5×10^5 cells	0.45	6,922 ± 1,345
4×10^5 cells	0.45	8,339 ± 596
3×10^5 cells	0.45	5,765 ± 2,063

TABLE (b)

PHA-Response of Naive Lymph Node Cells

Cells were obtained from BDFI mice.

A range of numbers of cells from 3 - 6 x 10^5 was tested in LTT.

PHA was used at 2 doses: 2.25 mg/ml and 0.45 mg/ml.

Determination of the Optimum Cell Number and Antigen Dose For OVA-Specific Transformation

A second set of test cultures were set up, this time using cells from lymph nodes draining the footpads of BDFI mice immunized 21 days previously with 100 µg OVA in FCA. Experiments on the DTH response of these mice had shown a peak of responsiveness 21 days after immunization.

In the first of these tests, the range of cell concentrations which had been found to respond well to PHA, that is 3×10^5, 4×10^5 and 5×10^5 cells per well, were tested against a range of doses of OVA, from 100 µg - 1 µg per well and the results are summarized in Table (c).

It was apparent from the lack of response, that despite the responsiveness of cells to a very low dose of a non-specific T cell mitogen, PHA, the antigen-specific response required a higher concentration of OVA before the OVA-specific population could respond.

The only indication of a response to OVA in this dose range was when the cells were at a concentration of 5×10^5 cells per well and so I decided to test this concentration first. In this culture, I tested the rsponse of 5×10^5 OVA-primed cells to a range of OVA concentrations between 10 µg and 1 000 µg per well and included a PHA-stimulated positive control. The results given in Table (d) produced a dose-response curve, which is shown in figure (a), with the optimum response to 250 µg OVA per well. I decided to utilise these optima for all subsequent cultures.

Confirmation of the T Cell-Dependency of OVA-Specific Transformation

In order to ensure that the lymphocyte transformation assay was a

Cells Per Well	μg OVA Per Well	Mean cpm ± s.e.
5×10^5	100	257 ± 81
5×10^5	50	6.3 ± 7
5×10^5	10	93 ± 71
5×10^5	1	22 ± 18
5×10^5	0	10, 8 (n = 2)
4×10^5	100	25 ± 21
4×10^5	50	17 ± 9
4×10^5	10	17 ± 9
4×10^5	1	13 ± 3
4×10^5	0	7, 45 (n = 2)
3×10^5	100	below background
3×10^5	50	13 ± 3
3×10^5	10	below background
3×10^5	1	below background
3×10^5	0	1 ± 2

TABLE (c)

Response of OVA-primed lymph node cells to OVA.

Cells were obtained from BDFI mice 21 days after immunization with OVA. 3, 4 or 5×10^5 cells were tested in LTT with a range of OVA doses as shown.

The response in wells without added OVA are expressed as individual values since there were not sufficient numbers of these wells to calculate mean cpm.

Added to 5×10^5 cells	Mean cpm ± s.e.	Stimulation Index
Nothing	158 ± 75	1
0.45 mg/ml PHA	8,269 ± 822	20.6
1,000 µg OVA	797 ± 240	5
750 µg OVA	1,246 ± 210	7.9
500 µg OVA	1,432 ± 233	9.1
250 µg OVA	1,820 ± 298	11.5
100 µg OVA	1,155 ± 168	7.3
50 µg OVA	755 ± 33	4.8
10 µg OVA	222 ± 26	1.4

TABLE (d)

Response of 5×10^5 OVA-primed Lymph Node Cells To OVA

Cells were obtained from BDFI mice 21 days after immunization with OVA.

5×10^5 cells were tested in LTT with a range of OVA doses as shown.

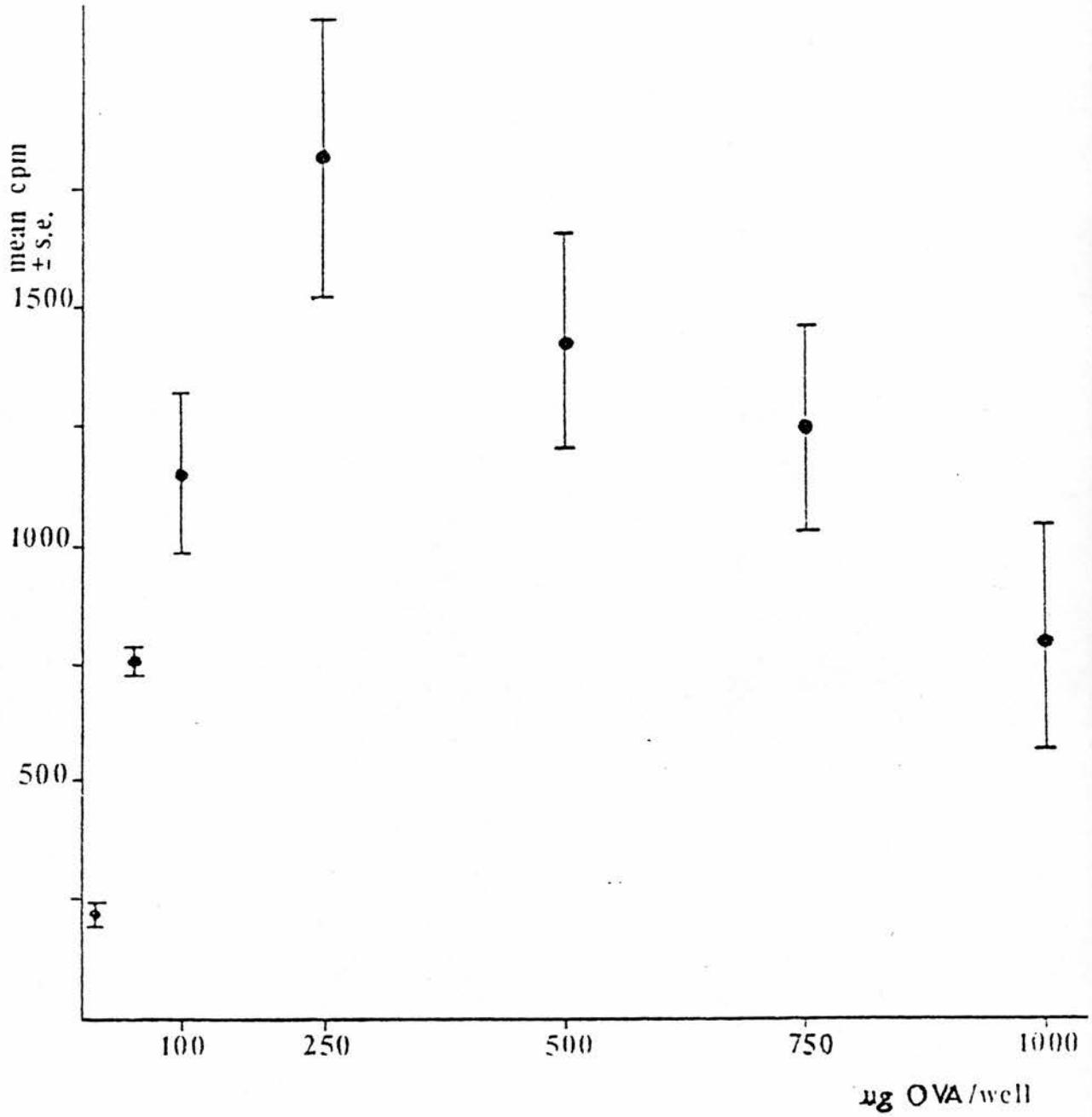


FIGURE (a)

Response of 5 x 10^5 OVA-primed lymph node cells to a range of doses of OVA.

Results are mean cpm ± s.e.. For responses in the dose range 10 µg - 250 µg, r = 0.97. For responses in the dose range 250 µg - 1000 µg, r = 0.97.

Cells were obtained from BDFI mice 21 days after immunization with OVA.

measure of antigen-stimulated T cell proliferation and to test that OVA was not behaving simply as a T cell mitogen, I performed a set of assays where in one case, T cells were removed from an OVA-primed lymph node population by incubation with anti-Thy 1.2 and complement as described at the beginning of this section.

In a second test, I examined the OVA-specific response of cells taken 3 weeks after a sham-immunization of saline in incomplete Freund's adjuvant. This illustrated the response of non-immune cells, with respect to OVA.

The absence of a response in the T-depleted lymph node cells (Table (e)) reveals the T cell dependency of the lymphocyte transformation assay. Removal of T cells effectively removed the response to OVA.

Finally, Table (f) illustrates that non-immunized mice do not respond to OVA <u>per se</u> and so I concluded that the assay effectively measured the T cell-dependent proliferation of OVA-primed cells in response to that specific antigen.

Culture Containing	Cell Pre-treatment	Mean cpm ± s.e.	Stimulation Index
Cells only	none	16 ± 5	1
PHA	none	1,975 ± 176	123.4
OVA	none	397 ± 36	24.8
Cells only	C, only	11 ± 9	1
PHA	C, only	1,067 ± 687	97
OVA	C, only	103 ± 69	9.4
Cells only	anti-Thy 1.2 + C	8 ± 4	1
PHA	anti-Thy 1.2 + C	22 ± 4	2.8
OVA	anti-Thy 1.2 + C	9 ± 3	1.1

TABLE (e)

Response of 5×10^5 OVA-primed lymph node cells to OVA and PHA: Effect of depleting T Cells.

Cells were obtained from BDFI mice 21 days after immunization with OVA.

T Cells were depleted from lymph node cell populations by treatment with anti-Thy 1.2 plus complement (C) as described in Chapter 2, Section (a).

Added to Culture	Mean cpm ± s.e.	Stimulation Index
Nothing	8 ± 4	1
PHA	613 ± 126	76.6
250 µg OVA	14 ± 6	1.8

TABLE (f)

OVA and PHA Response of 5×10^6 Lymph Node Cells From Mice Sham-Immunized with Saline in Incomplete Freund's Adjuvant.

BDFI mice were injected with 0.05 ml of a 1 : 1 emulsion of saline in adjuvant.

Cells were obtained 21 days after injection.

SECTION (c)

IMMUNOCHEMICAL TECHNIQUES

A. Preparation of Immunoadsorbent anti-OVA Antibodies Coupled to Sepharose (S4B-anti-OVA)

Anti-OVA Antibody

The source of anti-OVA antibodies was a rabbit anti-OVA hyperimmune antiserum (the generous gift of Dr Allan Mowat). The antiserum formed a single precipitin line in the presence of OVA antigen and 16% of the immunoglobulin (Ig) was specific anti-OVA. The Ig portion of the antiserum was isolated by repeated precipitation with a saturated ammonium sulphate solution and had a total protein content of 6.6 mg/ml. If 16% of this was anti-OVA antibody then the concentration of anti-OVA antibody in the Ig preparation was approximately 1 mg/ml.

Coupling Procedure

Anti-OVA Ig was coupled to Sepharose 4B (Cyanogen bromide-activated; Pharmacia Fine Chemicals) according to the manufacturer's recommendations laid out in their pamphlet "Affinity Chromatography". Briefly, the Ig preparation was dissolved in protein coupling buffer ($NaHCO_3$ (BDH), 0.1M, pH 8.3 containing NaCl (BDH), 0.5M) and added to Sepharose 4B gel (S4B) to a final concentration of 5 mg protein per ml of gel and mixed in an end-over-end mixer for 2 hours at room temperature. Excess protein was then washed away with fresh coupling buffer and the gel was mixed with ethanolamine solution

(Ch_2 $(OH)CH_2$ N_2 (BDH), IM, pH 9) for 2 hours at room temperature in order to block any remaining active sites on the S4B beads.

The gel was washed three times; first with coupling buffer, then with acetate buffer (0.1M pH , containing NaCl, 0.5M) and finally with coupling buffer. The final product was stored at $4^{\circ}C$ in PBS containg 0.2% sodium azide as a preservative.

Acetate buffer (1,000 ml):

CH_3COONa (BDH) 0.1N 16.6 ml

CH_3COOH (BDH) 0.1N 83.4 ml

In preparing S4B-anti-OVA, 2.65 ml of the Rabbit Ig was used to couple with 3.5 ml of S4B. Therefore 2.65 mg of anti-OVA Ig was added. According to the manufacturer's guidelines, the coupling procedure should be 90% efficient. This means that 2.4 mg of anti-OVA Ig (90% of 2.65 = 2.4) was coupled to 3.5 ml S4B. The concentration of anti-OVA Ig bound to S4B was therefore 690 µg per ml of S4B.

Binding Capacity of S4B-anti-OVA

In order to determine the OVA-binding capacity of the S4B-anti-OVA, 500 µl of a constant concentration of OVA (10 µg/ml) dissolved in normal mouse serum was added to increasing volumes of S4B-anti-OVA which was mixed for 4 hours at room temperature.

After this time, the S4B-anti-OVA was allowed to settle and the supernatant containing any unbound OVA was removed and tested for OVA concentration in the ELISA described in Chapter 2. The amount of OVA which remained bound to the S4B-anti-OVA was estimated using a "bead ELISA" which was developed by Mrs Hazel Drummond:

"Bead ELISA"

After incubation with OVA as above, the S4B-anti-OVA was washed 3 times in 5 - 10 ml ELISA washing solution by suspending the beads in the solution, allowing them to settle and removing the supernatant. The immuno-adsorbent beads were then mixed for 4 hours at room temperature in 0.1 ml of a 1 in 250 dilution of rabbit anti-OVA IgG conjugated to alkaline phosphatase (see Chapter 2, ELISA for OVA).

The supernatant was removed and the beads were once again washed 3 times with ELISA washing solution. ELISA substrate solution (1 ml) was then added and the beads were mixed with this until the coloured reaction product had developed. Aliquots of bead supernatant containing the colour were removed into an ELISA plate and read in the automatic ELISA reader at wavelength 405 nm.

The results (summarized in figure (b) and Table (g)) show that as the amount of S4B-anti-OVA was increased relative to a fixed amount of OVA, the amount of OVA left unbound in the supernatant decreased and the amount of OVA bound to S4B-anti-OVA showed a corresponding increase.

After mixing 500 µl of 10µg/ml OVA with 200 µl S4B-anti-OVA, only 18 ng OVA remained in the supernatant of the original 5000 ng added. This meant that 200 µl S4B-anti-OVA had effectively bound 4982 ng OVA, approximately 5 µg. In my experience, the concentration of OVA found in serum 1 hour after a feed of 25 mg was usually of an order of 10 ng and so I chose to use 200 µl of S4B-anti-OVA, with a binding capacity for approximately 5 µg OVA in my subsequent immune-adsorption studies.

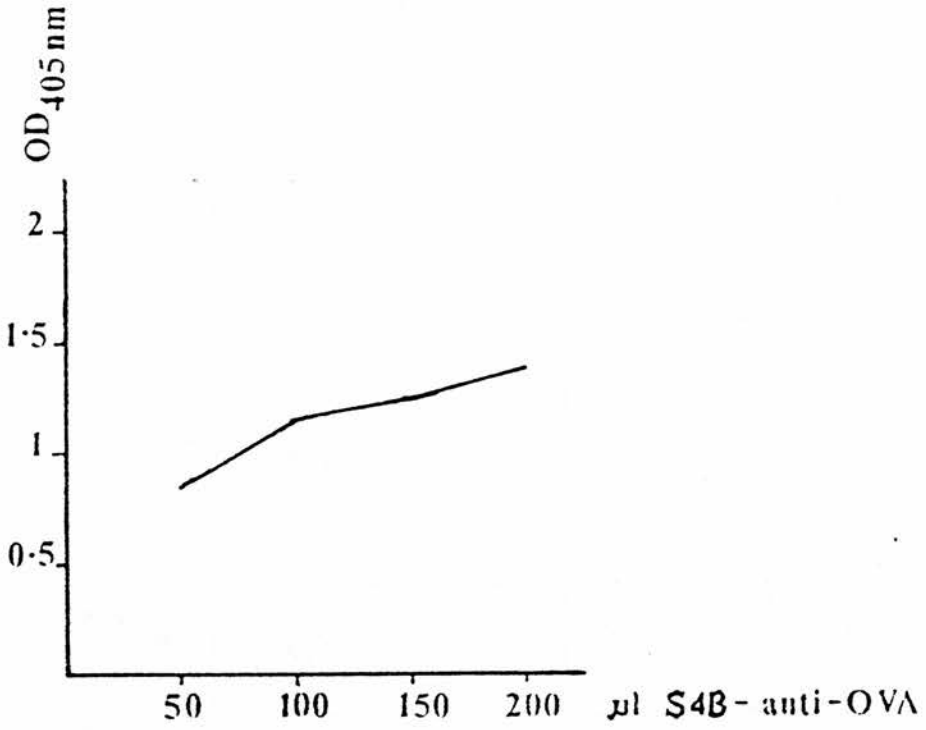


FIGURE (b)

OVA bound to S4B-anti-OVA detected by "bead" ELISA expressed as O.D. 405 nm.

Increasing amounts of IgG anti-OVA coupled to Sepharose 4B were added to 5 µg OVA. The amount of OVA bound by the Sepharose beads increases as more beads are added.

Volume of S4B-Anti-OVA	O.D. 405 nm	OVA per ml
50 µl (i)	0.714	1.8 µg
50 µl (ii)	0.668	0.9 µg
100 µl (i)	0.340	234 ng
100 µl (ii)	0.302	200 ng
150 µl (i)	0.071	45 ng
150 µl (ii)	0.065	40 ng
200 µl (i)	0.055	35 ng
200 µl (ii)	0.052	38 ng
NMS (i)	0.012	neg
blank	0.018	neg
conjugate blank	0.013	neg

TABLE (g)

OVA (by ELISA) In Supernatants After S4B-Anti-OVA Treatment

Increasing volumes of S4B-Anti-OVA were added to 500 µl normal mouse serum containing 5 µg OVA. Each sample was run in duplicate. The test included a "blank" containing only ELISA substrate, a "conjugate blank" containing rabbit anti-OVA IgG conjugated to alkaline phosphatase plus ELISA substrate, and a sample of normal mouse serum (NMS). The amount of OVA remaining in supernatant decreases as more S4B-Anti-OVA is added.

B. Standardisation of a Gel Filtration Column

Equipment and Running Conditions

Serum proteins were fractionated by gel filtration through a column of Sephacryl S200, Superfine (Pharmacia Fine Chemicals) which separated proteins on the basis of molecular weight.

The column was a Wright glass column which was 60 cm long with internal diameter 22 mm (Amicon). According to the manufacturer's guidelines for the use of Sephacryl, the column was packed with S200 in 0.25M NaCl with 0.2% sodium azide. The sodium chloride gel buffer was de-gassed before use.

After packing, the total volume of gel in the column, the bed volume, was 151 ml. Samples of between 3% and 4% of this volume (about 5 ml) were routinely applied to the base of the column and passed upwards through the gel at 1.4 ml/minute by means of a peristaltic pump. Fractions coming off the column were continuously monitered by a "flow-through" spectrophotometer (LKB Uvichord II) set at 208 nm wavelength and connected to a recorder (Servoscribe, Belmont Instruments). Fractions of 3 ml were collected sequentially in an LKB 2070 Ultrorac II fraction collector.

Calibration of the Column

The column was calibrated using a mixture of molecules of known molcular weights. The void volume (Vo) of the column was determined by applying Blue Dextran to the column. This large molecule (Molecular Weight = 2,000,000) is not retained by Sephacryl beads which exclude molecules of weight greater than 250,000 and appears in the volume of buffer flowing ahead of the filtered sample.

The calibration mixture contained:

Column Buffer		5ml	
Blue Dextran	(Pharmacia)	MW = 2,000,000	1mg
Ovalbumin	(Sigma)	MW = 43,500	100 mg
Cytochrome - c	(Sigma)	MW = 12,400	5 mg
glycyl tyrosine	(Sigma)	MW = 238	10 mg

The di-peptide glycyl-tyrosine was eluted last from the column and its elution volume was considered the total working volume (Ve) of the column. The elution profiles of these standards are shown in figure (c). The elution volume (Ve) of each standard was used to calculate a distribution coefficient Kav from the formula:

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Kav represents the fraction of the gel volume which is available for diffusion of a given solute species and should therefore have a linear relationship with molecular weight. When Kav was calculated for each standard and compared to molecular weight by linear regression analysis a correlation co-efficient of r = 0.93 was found. This demonstrates a good linear relationship between Kav and $\log_{10}$ molecular weight, and indicates that the column was functioning efficiently as a molecular "sieve".

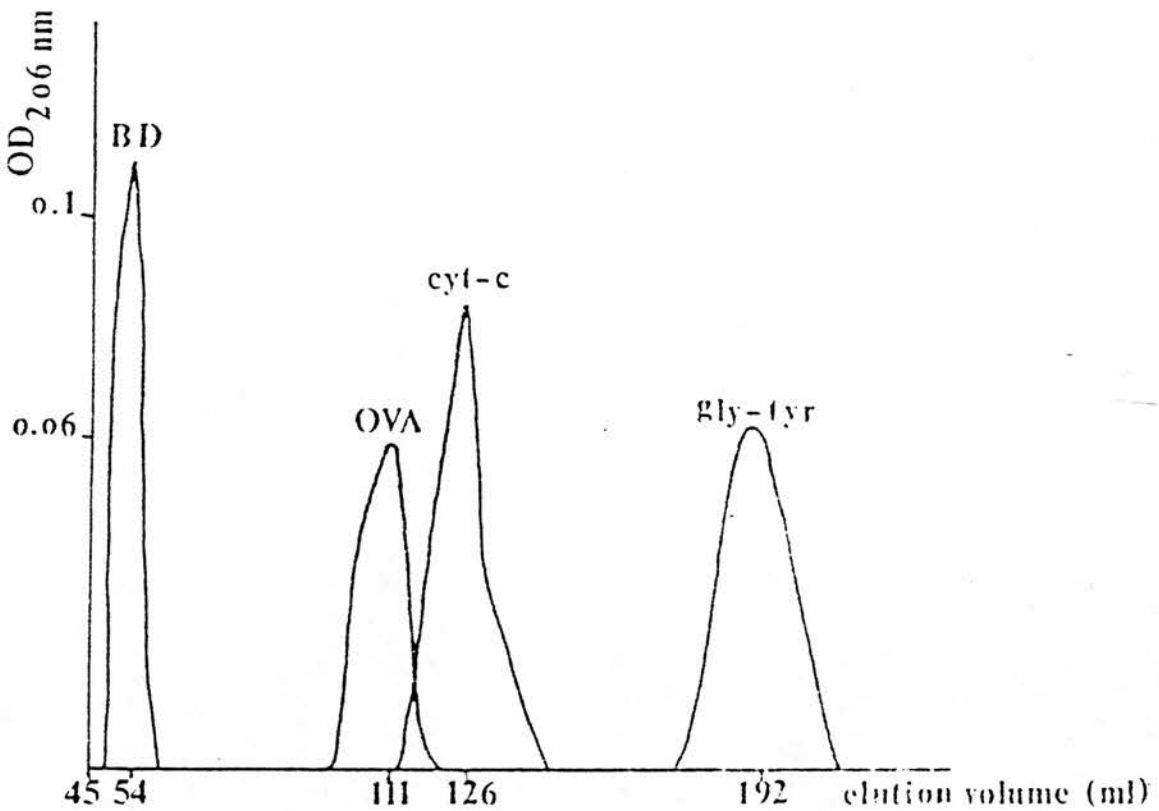


FIGURE (c)

Calibration of gel filtration column.

Standard solutions of Blue Dextran (BD), OVA, cytochrome-c (cyt-c) and glycyl tyrosine (gly-tyr) were used to calibrate the column. Elution volumes illustrate where peak protein concentrations are found. These are expressed as OD 206 nm.

CHAPTER 3

STUDIES IN VITRO OF IMMUNE REGULATION BY ANTIGEN FEEDING

SECTION (a): THE INDUCTION OF SYSTEMIC TOLERANCE TO OVALBUMIN BY FEEDING

3.1 Introduction

It had been previously demonstrated in this laboratory that a single feed of 25 mg OVA caused suppression of subsequent immune responses to OVA in BALB/c mice and that tolerance to OVA induced in this manner is CY-sensitive, indicating the involvement of suppressor T cells in its mechanism (Mowat et al, 1982). When the CY-sensitive mechanism is removed prior to OVA feeding, a local cell-mediated immune response to OVA is induced in the small-intestinal mucosa and the mesenteric lymph nodes (Mowat and Ferguson, 1981 a).

These experiments were performed either in vivo or by assaying the in vitro inhibition of lymphocyte migration as a test for cell-mediated immunity. My research aim at this stage was to study cellular interactions associated with the induction of oral tolerance and so I utilised in vitro antigen-driven transformation of lymphocytes as a test for local cell-mediated immunity.

I followed the hypothesis that removal of a CY-sensitive suppressor mechanism led to the abrogation of oral tolerance to subsequent antigen feeding and permitted the induction of cell-mediated immunity in the gut. I reasoned that the mesenteric lymph nodes of such animals should contain activated lymphocytes capable of transforming in vitro in response to OVA. If such a cellular immune response was identified it could then be analysed and characterised further.

Having established a mouse lymphocyte transformation test or LTT,

as an *in vitro* assay of T cell-dependent antigen-induced stimulation of primed lymph node cells (Chapter 2, Section (b)), I first of all repeated standard *in vivo* experiments to show the induction of oral tolerance to OVA and its abrogation by CY injected prior to OVA feeding. In parallel with these experiments, I assessed the LTT as an assay for systemic cell-mediated immunity, its suppression by antigen feeding and the effect of CY on that suppression.

In a second set of experiments, described in section (b) of this chapter, I studied in more detail the kinetics of the *in vitro* response to OVA of cells in the mesenteric lymph nodes of CY-treated mice by assaying lymphocyte transformation to OVA at various times after feeding a single tolerizing dose of OVA to CY-treated mice. Separate experiments were also set up to assess lymphocyte activation in peripheral and mesenteric lymph nodes on different days following OVA feeding, again with a single tolerizing dose of OVA.

3.2 Design of Experiments

Groups of BDFI mice were treated according to the experimental protocol in figure 1 and fed on day 0 of the experiment with either 0.2 ml physiological saline (Group 1) or 25 mg OVA (Group 2) by intragastric intubation. A third group was injected i.p. with 100 mg/kg CY on day -2, that is 2 days before being fed 25 mg OVA (Group 3). One week after feeding, on day 7 of the experiment, all mice were given a primary immunization with 100 µg OVA in Freund's complete adjuvant (FCA) and 3 weeks after immunization, serum anti-OVA antibodies were measured using a haemagglutination assay. Antibody which was sensitive to mercaptoethanol dissociation was classed as IgM and non-sensitive as IgG. The same mice were also tested for delayed

hypersensitivity by skintesting with antigen in the footpad and measuring the 24 hour increment in footpad thickness.

For the parallel in vitro experiment, groups of mice were treated according to the same experimental protocol. Three weeks after immunization they were killed and cells from mesenteric (MLN) and peripheral (popliteal and inguinal) lymph nodes (PLN) were prepared for 5 day cultures. Cultures consisted of quadruplicate wells containing 5×10^5 cells in supplemented RPMI 1640 medium to which were added either a T cell mitogen, phytohaemagglutinin (PHA) as a control to show positive transformation, or 250 µg OVA or nothing. The cultures were labelled on day 4 with 0.01 µCi C^{14}-thymidine and harvested on day 5 for β-scintillation counting. In every culture, 4 spare wells were not labelled and were used to determine background radiation which was subtracted from the counts per minute (cpm) in labelled wells. The mean cpm was calculated and a stimulation index obtained by dividing the mean cpm of PHA or OVA-containing wells by that found in unstimulated wells. Students t-test was used to compare quadruplicates within groups only. Cultures for each experimental group were set up on separate days owing to the amount of work and length of time required for each culture.

3.3 Suppression of Serum Antibody Responses by Feeding Ovalbumin

The anti-OVA response of mice 3 weeks after an immunization with 100 µg OVA in FCA injected into the footpad is shown in figure 2. The mean haemagglutinating antibody titre of mice fed 25 mg OVA 7 days before OVA immunization (Group 2) is significantly suppressed compared to mice which were fed saline and then immunized (Group 1 versus Group 2, $P < 0.01$).

GROUP	DAY -2	DAY 0	DAY 7	DAY 27	DAYS 28 - 29
1	-	Fed Saline	OVA Immunized	Bleed	DTH Test or LTT
2	-	Fed OVA	OVA Immunized	Bleed	DTH or LTT
3	CY i.p.	Fed OVA	OVA Immunized	Bleed	DTH or LTT

FIGURE 1

Experimental protocol to test the induction of tolerance to ovalbumin by feeding and the effect of cyclophosphamide pre-treatment.

CY was injected at 100 mg/kg dose.

Mice were fed either 25 mg OVA dissolved in 0.2 ml Sal or 0.2 ml Sal only.

Mice were immunized with 100 μg OVA in CFA.

DTH was elicited in a footpad by injection of 100 μg OVA dissolved in 0.05 ml saline.

3.4 Suppression of Delayed Hypersensitivity by Feeding Ovalbumin

Shown also in figure 2, a single feed of 25 mg OVA induces suppression of the DTH response to a subsequent immunization with OVA in FCA compared to the normal response of mice fed saline prior to immunization (Group 1 versus Group 2, $P < 0.05$).

3.5 The Effect of Cyclophosphamide on Systemic Immunity after OVA Feeding

A dose of 100 mg/kg CY injected i.p. 2 days before OVA feeding abrogated the suppression of DTH induced by OVA feeding and a normal DTH response was seen in this group (figure 2, group 3). However, in the same mice given CY pretreatment, the serum antibody response remained suppressed. This observation contrasts with the effect of CY in BALB/c mice. Under identical experimental conditions, CY abrogates tolerance of both antibody and DTH responses in BALB/c mice (Mowat *et al*, 1982). This abrogation of only DTH tolerance by CY in BDFI mice has been confirmed independently by Strobel and by Hanson (personal communications, 1982).

3.6 Proliferative Responses of Lymph Node Cells in vitro: Effects of OVA Feeding and CY Pretreatment

The results obtained by lymphocyte transformation assays are given in Table 1. The group numbers correspond to those of the experimental protocol in figure 1. That is, Group 1 were immunized with 100 µg OVA in FCA. This treatment previously induced a positive DTH result *in vivo* (figure 2) and the *in vitro* LTT shows the presence of antigen-driven stimulation in the draining peripheral lymph nodes

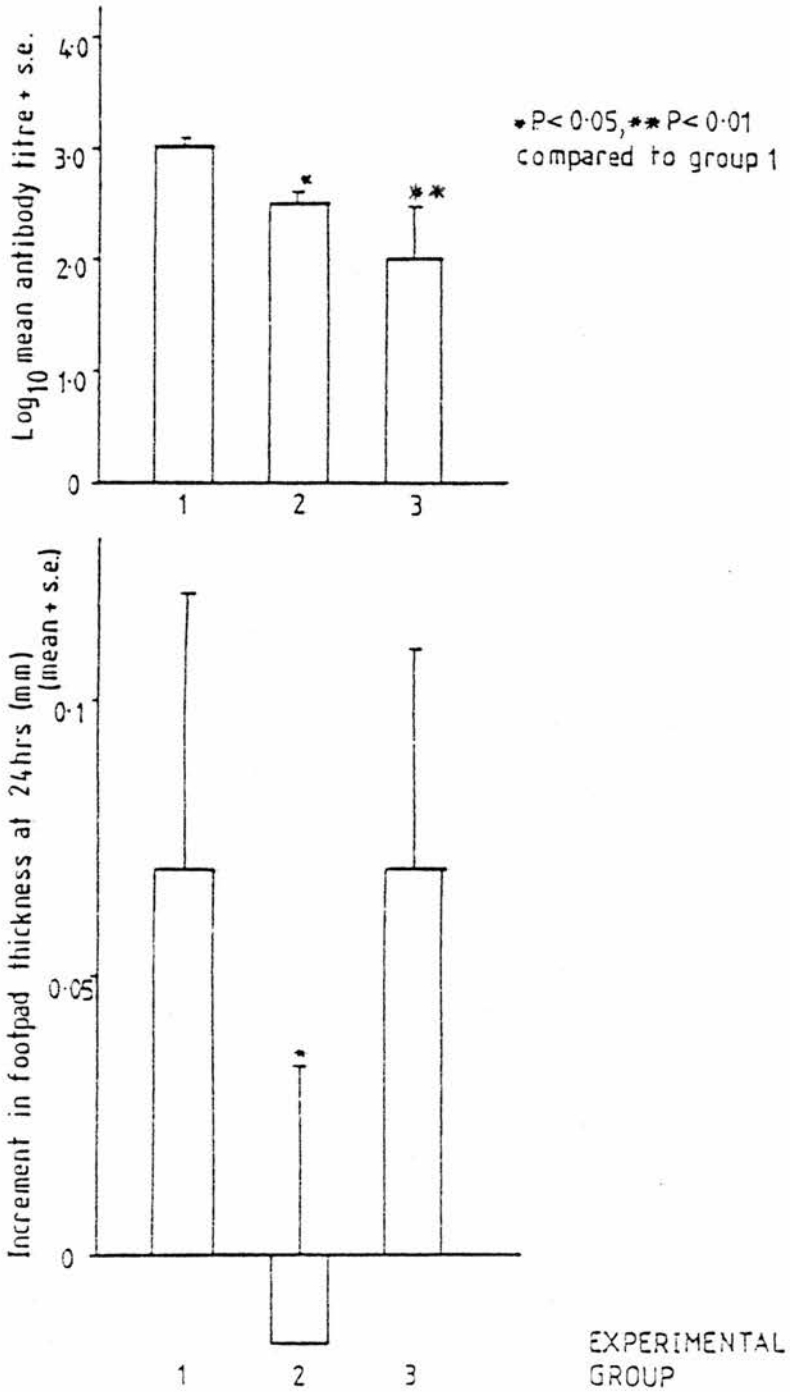


FIGURE 2

Systemic Humoral and Cell-Mediated Responses of BDFI Mice Fed Either Saline or OVA One Week Before Immunization and the Effect of Cyclophosphamide Prior to OVA Feeding.

Experimental groups as referred to in Figure 1: 1; saline fed, 2; OVA fed, 3; CY treated, OVA fed.

Antibody was measured by indirect haemagglutination.

DTH was measured as footpad swelling 24 hrs after antigen challenge.

Responses were assayed 21 days after OVA immunization.

of identically treated mice ($P < 0.001$, OVA-containing compared to unstimulated wells). No stimulation was seen in cells from mesenteric lymph nodes.

Group 2 shows the *in vitro* response of cells from mice fed OVA and then OVA-immunized. This treatment caused suppression of DTH *in vivo* (figure 2), however, primed cells are still present in the draining lymph nodes of these animals and respond to OVA *in vitro* (cells with OVA versus unstimulated wells, $P < 0.001$). Mesenteric lymph nodes did not respond to OVA in this test.

Group 3 is the response of CY-treated OVA-fed mice which show a positive result in a DTH skintest due to the abrogation of oral tolerance. The stimulation index of peripheral lymph node cells with OVA shows OVA-driven transformation and indeed the mean cpm is significantly higher than unstimulated cells ($P < 0.001$). However, the counts obtained are low overall compared to groups 1 and 2 and illustrate the variability of this test. The mesenteric lymph node cells responded to OVA sufficiently to cause a significant increase in mean cpm ($P < 0.005$, stimulation index = 2.6) but this in turn was still significantly lower than the response of peripheral node cells ($P < 0.001$).

3.7 Conclusions and Discussion of Section (a)

Lymphocyte transformation in response to OVA *in vitro* correlates with the presence of a DTH response in mice immunized with OVA in adjuvant and has been previously reported by Miller and Hanson, 1979. This also extends the observations of Mowat (Mowat and Ferguson, 1981) that inhibition of migration of lymphocytes *in vitro* in the presence of OVA is a marker for CMI reactions.

Group	Culture Containing	Mean cpm ± s.e. (Stimulation index)	P Value, compared to Unstimulated	P Value, compared to MLN + OVA
1	PLN + PHA	1292 ± 113 (38)	< 0.001	-
	PLN + OVA	2869 ± 855 (84.4)	< 0.005	< 0.005
	PLN	34 ± 27 (1)	-	-
	MLN + PHA	1747 ± 564 (103)	< 0.005	-
	MLN + OVA	25 ± 8 (1.5)	N. S.	-
	MLN	17 ± 2 (1)	-	-
2	PLN + PHA	514 ± 167 (12.5)	< 0.005	-
	PLN + OVA	1261 ± 177 (30.8)	< 0.001	< 0.001
	PLN	41 ± 27 (1)	-	-
	MLN + PHA	1041 ± 259 (43.4)	< 0.001	-
	MLN + OVA	20 ± 11 (0.8)	N. S.	-
	MLN	24 ± 13 (1)	-	-
3	PLN + PHA	790 ± 188 (21.4)	< 0.001	-
	PLN + OVA	634 ± 104 (17.1)	< 0.001	< 0.001
	PLN	37 ± 8 (1)	-	-
	MLN + PHA	204 ± 165 (29.1)	N. S.	-
	MLN + OVA	18 ± 2 (2.6)	< 0.005	-
	MLN	7 ± 3 (1)	-	-

TABLE 1

Proliferative Responses of OVA-Immunized Mice: Effects of OVA Feeding and CY Pretreatment.

Experimental groups as referred to in Figure 1: 1; saline fed, 2; OVA fed, 3; CY treated, OVA fed.

Cells were collected from BDFI mice 21 days after immunization with OVA

Suppression of the *in vitro* proliferative response of PLN cells after antigen feeding and immunization has been previously reported by other workers (Miller and Hanson, 1979; Silverman *et al*, 1983). In my experiments using assays of this kind, I was unable to detect suppression of the proliferative response in mice with suppressed DTH responses, for example those mice which were fed OVA prior to immunization. I tested lymphocyte transformation at 21 days after immunization. This was in contrast to Miller and Hanson who tested at 8 days after immunization and Silverman *et al* who used a similar protocol for BSA. However, the *in vitro* inhibition of migration of lymphocytes taken from the draining lymph nodes of mice immunized with OVA in FCA injected into the footpad, developed in this laboratory by Dr Allan Mowat, revealed no OVA-specific inhibition of migration before 3 weeks following OVA immunization and this was influential in my decision to test for activated cells from the same source at this time. Mowat observed that production of a lymphocyte migration inhibition factor did not always correlate with the presence of DTH, (Mowat and Ferguson, 1981). It is not too surprising therefore, that different regimes of immunization and sampling may highlight different phases of the highly regulated response to a fed protein antigen.

I used slightly different culture conditions from the 2 groups of workers mentioned above, labelling cells with C^{14} instead of tritium for example, and using a different number of cells per well cultured in supplemented RPMI 1640. These conditions were based on my preliminary experiments determining the optimum conditions for a proliferative response using cells from the draining lymph nodes of mice immunized with OVA in FCA.

The response of CY-treated, OVA-fed and immunized mice (Table 1,

group 3) is difficult to interpret. Peripheral nodes produce a positive response to OVA although the degree of this response relative to DTH is impossible to tell from this experiment. The mesenteric lymph nodes also exhibit low grade transformation although this was much less than the peripheral lymph node cells. It seemed feasible that the systemic injection of antigen in Freund's complete adjuvant produced an exaggerated peripheral response compared to that in the mesenteric lymph nodes which were not adjuvant stimulated. I therefore decided to study the in vitro responses of lymphocytes from mice which were fed antigen and not systemically immunized. These experiments are described in section (b) of this chapter.

SECTION (b): LOCAL CMI REACTIONS OF PERIPHERAL AND MESENTERIC LYMPH NODE CELLS FOLLOWING OVALBUMIN FEEDING: EFFECTS OF CYCLOPHOSPHAMIDE PRETREATMENT

3.8 Lymphocyte Transformation One Week After OVA Feeding and the Effect of Cyclophosphamide

Previous work by Dr Mowat in this laboratory had shown that cells from the mesenteric lymph nodes of mice injected with 100 mg/kg CY 2 days prior to OVA feeding showed in vitro inhibition of lymphocyte migration in the presence of OVA. This response developed within 24 hours of a single OVA feed and persisted for 14 days. Mice fed OVA without previous CY injection did not exhibit this response (Mowat and Ferguson, 1981).

I studied local immunity based on the cyclophosphamide model using LTT instead of migration inhibition and comparing the responses of mesenteric and peripheral lymph nodes within the same animals to

determine whether a true local immunity developed, for example, in the mesenteric lymph nodes without concomitant peripheral activation.

A single time point was selected for initiation of the LTT at 7 days after feeding 25 mg OVA. This single feed on day 0 was previously sufficient to induce oral tolerance (section 3.2). Seven days after feeding was the usual interval before systemic challenge to measure in vivo suppression and also came midway in the time after feeding when specific migration inhibition of lymphocytes could be detected in CY-treated mice. Lymphocyte transformation was assessed in mice given 100 mg/kg CY at 9 days before culture and also in mice given either a single feed of 25 mg OVA 7 days before culture. The experimental protocol is summarised in figure 3.

In these experiments, where mice were not systemically immunized with antigen in adjuvant, the peripheral lymph nodes were small and so in order to have enough cells for culture, both popliteal nodes and both inguinal lymph nodes were taken. As before, each experimental group was set up as a separate culture.

The antigen dose-response to OVA had been determined using peripheral lymph nodes. As a precaution that the optimum OVA dose for transformation of mesenteric lymph node cells might vary slightly, group 4 was set up for 3 different doses of OVA around the optimum dose for peripheral stimulation. That is, 200 µg, 250 µg and 300 µg per well.

The results of this experiment are summarised in table 2. Group 6 which had been injected with CY only showed no in vitro response to OVA after 9 days either in peripheral or mesenteric lymph nodes. The background count in unstimulated wells was observably higher in cells from CY-treated mice than in those which were not given CY (group 5).

GROUP	DAY -2	DAY 0	DAY 7
4	CY i.p.	Fed OVA	LTT
5	-	Fed OVA	LTT
6	CY i.p.	-	LTT

FIGURE 3

Experimental protocol to test lymphocyte transformation 7 days after feeding and the effect of cyclophosphamide pretreatment. CY was injected at a dose of 100 mg/kg. OVA was fed at a dose of 25 mg in 0.2 ml Sal.

Group	Culture Containing	Mean cpm ± s.e. (Stimulation Index)	P, value, compared to unstimulated
4	PLN + PHA	1194 ± 338 (2.8)	< 0.025
	PLN + OVA	96 ± 14 (0.2)	< 0.005
	PLN	424 ± 126 (1)	-
	MLN + PHA	2493 ± 531 (18.2)	< 0.001
	MLN + OVA (300 µg)	653 ± 275 (4.8)	< 0.02
	MLN + OVA (250 µg)	986 ± 589 (7.2)	N. S.
	MLN + OVA (200 µg)	1147 ± 224 (8.4)	< 0.001
	MLN	137 ± 29 (1)	-
5	PLN + PHA	1318 ± 568 (3295)	< 0.01
	PLN + OVA	7 ± 2 (17.5)	< 0.05
	PLN*	0.4 ± 3.5 (1)	-
	MLN + PHA	1305 ± 68 (62.1)	< 0.001
	MLN + OVA	55 ± 17 (2.6)	< 0.05
	MLN	21 ± 13 (1)	-
6	PLN + PHA	1354 ± 231 (6.6)	< 0.001
	PLN + OVA	249 ± 77 (1.2)	N. S.
	PLN	205 ± 86 (1)	-
	MLN + PHA	1476 ± 263 (6.8)	< 0.001
	MLN + OVA	134 ± 43 (0.6)	N. S.
	MLN	217 ± 65	-

TABLE 2

Proliferative Responses of Non-Immunized Mice: Effects of OVA Feeding ± CY Pretreatment.

Experimental groups as referred to in Figure 3: 4; CY treated, OVA fed, 5; OVA fed, 6; CY treated only.

Mice were injected with CY 2 days prior to OVA feeding.

Cells were obtained from BDFI mice 7 days after OVA feeding or 9 days after CY treatment (2 days + 7 days)

The mesenteric lymph node response of OVA-fed mice (group 5) was sufficient to cause a significant increase in cpm ($P < 0.05$ compared to unstimulated wells) although the stimulation index only reached 2.6. The background response of peripheral lymph node cells in group 5 was unusually low (0.4 ± 3.5). This produced artificially high stimulation indices and a significant difference in mean cpm of OVA-stimulated wells. This is pseudo-stimulation and is not the result of transformation in the test wells since the mean cpm obtained with OVA was only 7 ± 2.

Group 4 which were CY-treated and OVA fed also showed higher background counts than group 5 which had not been CY-injected. Mesenteric node cells with 300 µg and 200 µg OVA added had a significant increase in mean cpm compared to unstimulated wells. The 250 µg OVA dose caused an increase in mean cpm which was not significant due to the large standard error of the mean which was the result of well-well variation. Nonetheless the optimum OVA dose for mesenteric lymph node cells seems to be the same as for peripheral cells.

Cells from peripheral nodes in group 4 did not respond to OVA and the counts obtained were even lower than background on this occasion.

Because of the apparent stimulation in mesenteric lymph node cells following CY injection and OVA feeding, this culture group (group 4) was repeated twice further. The results of these repeat test are given in table 3.

In both subsequent tests, no OVA-specific stimulation of mesenteric lymph node cells was found. The stock antigen solution used in all three cultures was tested by the Microbiology Department in the Western General Hospital and found free of contamination by

Group	Culture Containing	mean cpm ± s.e. (stimulation index)	P value, compared to unstimulated
4 (2nd test)	PLN + PHA	991 ± 435 (8.6)	< 0.02
	PLN + OVA	357 ± 116 (3.1)	N. S.
	PLN	115 ± 12 (1)	-
	MLN + PHA	956 ± 281 (10.8)	< 0.005
	MLN + OVA	95 ± 47 (1.1)	N. S.
	MLN	88 ± 11 (1)	-
4 (3rd test)	PLN + PHA	1603 ± 831 (69.6)	< 0.02
	PLN + OVA	51 ± 8 (2.2)	< 0.02
	PLN	23 ± 12 (1)	-
	MLN + PHA	1551 ± 235 (24.6)	< 0.001
	MLN + OVA	80 ± 23 (1.3)	N. S.
	MLN	63 ± 4 (1)	-

TABLE 3

Proliferative Responses of Non-Immunized Mice: Repeated Tests in CY Treated OVA Fed Mice

Experimental groups as referred to in Figure 3.

Cells were obtained from BDFI mice 7 days after OVA feeding. Mice were injected with CY 2 days prior to OVA feeding.

bacteria thus ruling out the possibility of false counts being obtained in the first test due to either proliferating bacteria or lymphocyte stimulation in response to bacterial products.

I decided to run a time-course of cultures from CY-treated OVA-fed mice commencing 1 day after feeding and continuing daily for 6 days. I reasoned that the low grade mesenteric lymph node response seen in the first test at 7 days after feeding, may have been residual activity indicative of a response occurring earlier and so might not always be above the limits of detection of my assay at 7 days. I also followed the in vitro response of peripheral lymph node cells at different times after OVA feeding only, and compared the responses where possible with those of mesenteric lymph nodes from the same animals.

3.9 Time-Course of Lymphocyte Transformation in CY-Treated OVA-Fed Mice

BDFI mice were injected i.p. with 100 mg/kg CY and fed 25 mg OVA 2 days later. Groups of 5 to 6 mice were then killed daily for up to 6 days after feeding and had mesenteric and peripheral lymph node cells cultured. The results of these cultures are combined in Table 4.

Cells taken from peripheral lymph nodes at 1 day after OVA feeding showed a significant increase in cpm when cultured with OVA compared to control wells ($P < 0.025$) and had a stimulation index of 3.7. Mesenteric lymph node cells had a stimulation index of 4 in the presence of OVA but did not show a significant increase in mean cpm ($P < 0.2$). No antigen-specific activity was seen in cultures initiated at 2, 3 and 4 days after OVA-feeding with CY pretreatment.

Days after OVA Feeding	Culture Containing	Mean cpm ± s.e. (stimulation index)	P value, compared to unstimulated
1	PLN + PHA	712 ± 154 (28.5)	< 0.001
	PLN + OVA	93 ± 38 (3.7)	< 0.025
	PLN	25 ± 10 (1)	-
	MLN + PHA	726 ± 228 (66)	< 0.005
	MLN + OVA	44 ± 31 (4)	N. S.
	MLN	11 ± 4 (1)	-
2	PLN + PHA	554 ± 184 (19.7)	< 0.005
	PLN + OVA	32 ± 14 (1.1)	N. S.
	PLN	28 ± 17 (1)	-
	MLN + PHA	1054 ± 59 (45.8)	< 0.001
	MLN + OVA	29 ± 5 (1.3)	N. S.
	MLN	23 ± 14 (1)	-
3	PLN + PHA	776 ± 283 (17.6)	< 0.02
	PLN + OVA	33 ± 13 (0.75)	N. S.
	PLN	44 ± 9 (1)	-
	MLN + PHA	318 ± 56 (4)	< 0.001
	MLN + OVA	27 ± 14 (0.34)	< 0.01
	MLN	79 ± 16 (1)	-
4	PLN + PHA	1309 ± 533 (32.7)	< 0.02
	PLN + OVA	55 ± 9 (1.4)	N. S.
	PLN	40 ± 4 (1)	-
	MLN + PHA	2294 ± 422 (11.9)	< 0.001
	MLN + OVA	250 ± 112 (1.3)	N. S.
	MLN	193 ± 19 (1)	-
5	PLN + PHA	3717 ± 520 (56.3)	< 0.001
	PLN + OVA	349 ± 190 (5.3)	< 0.05
	PLN	66 ± 41 (1)	-
	MLN + PHA	1192 ± 637 (3.5)	N. S.
	MLN + OVA	870 ± 273 (2.6)	< 0.025
	MLN	338 ± 64 (1)	-
6	PLN + PHA	1769 ± 1130 (12.8)	N. S.
	PLN + OVA	265 ± 67 (1.9)	N. S.
	PLN	138 ± 82 (1)	-
	MLN + PHA	2808 ± 211 (14.9)	< 0.001
	MLN + OVA	1406 ± 548 (7.5)	< 0.02
	MLN	188 ± 52 (1)	-

TABLE 4

Proliferative Responses of Non-Immunized Mice: Time-course of Response Following CY Treatment and OVA Feeding.

BDFI mice were injected with CY 2 days prior to OVA feeding.

Cells were obtained at various times following OVA feeding.

On the 5th day after OVA feeding, both peripheral and mesenteric lymph node cells showed a significant increase in cpm in response to OVA (PLN $P < 0.05$; MLN, $P < 0.025$) with stimulation indices of 5.3 and 2.6 respectively. Six days after feeding the response in mesenteric lymph node cells had continued to rise and the stimulation index was now 7.5 with mean cpm significantly greater than control wells without OVA ($P < 0.02$). This result is similar in degree to the one previous culture of mesenteric lymph nodes (Group 4, table 2) at 7 days after OVA-feeding in CY-treated mice which showed OVA-driven transformation. The peripheral lymph node culture 6 days after feeding produced a disappointing result with large variation between wells such that no significant difference was found in either PHA or OVA-stimulated cultures.

3.10 Lymphocyte Transformation Following OVA Feeding

Mice were fed 25 mg OVA on day 0 and on days 1, 3, 5, 7 and 14, groups of mice were used as a source of cells for cultures. The results of these cultures are summarised in table 5. Cells from peripheral and mesenteric lymph nodes collected 1 day after OVA feeding showed *in vitro* responsiveness to OVA, having a significantly increased mean cpm compared to unstimulated cells (PLN, $P < 0.05$; MLN, $P < 0.05$), however, the response was quite low-grade with stimulation indices of 1.9 and 2.6 in peripheral and mesenteric nodes respectively. A similar low-grade response was found in peripheral lymph nodes only, at 3 days after feeding and this was absent at 5 days.

More than one test was run at 7 days after feeding and the first test revealed no response in mesenteric lymphocytes. This is unlike the 7 day control group which were fed OVA only in the experiment

Days after OVA feed	Culture containing	Mean cpm ± s.e. (stimulation index)	P value, compared to unstimulated
1	PLN + PHA	674 ± 317 (25.9)	0.02
	PLN + OVA	49 ± 9 (1.9)	0.05
	PLN	26 ± 8 (1)	-
	MLN + PHA	981 ± 208 (37.7)	0.001
	MLN + OVA	67 ± 21 (2.6)	0.05
	MLN	26 ± 11 (1)	-
3	PLN + PHA	2510 ± 57 (31.8)	0.001
	PLN + OVA	32 ± 9 (2)	0.005
	PLN	16 ± 5 (1)	-
	MLN + PHA	1110 ± 132 (30)	0.001
	MLN + OVA	42 ± 6 (1.1)	N. S.
	MLN	37 ± 10 (1)	-
5	PLN + PHA	829 ± 216 (27.6)	0.001
	PLN + OVA	44 ± 14 (1.5)	N. S.
	PLN	30 ± 11 (1)	-
	MLN	Not determined	
7 (1st test)	PLN + PHA	270 ± 113 (2.7)	= 0.05
	PLN + OVA	108 ± 26 (1.1)	N. S.
	PLN	100 ± 57 (1)	-
	MLN + PHA	821 ± 86 (25.6)	0.001
	MLN + OVA	30 ± 4 (0.9)	N. S.
	MLN	32 ± 10 (1)	-
7 (2nd test)	PLN + PHA	1055 ± 367 (34)	0.01
	PLN + OVA	197 ± 84 (6.4)	0.02
	PLN	31 ± 10 (1)	-
	MLN	Not determined	
14	PLN + PHA	1473 ± 387 (25.8)	0.001
	PLN + OVA	262 ± 95 (4.6)	0.01
	PLN	57 ± 9 (1)	-
	MLN	Not determined	

TABLE 5

Proliferative Responses of Non-Immunized Mice Following OVA Feeding.

Cells were obtained from BDFI mice at various times following OVA feeding.

including CY-treated mice (Group 5, table 2). In this case, OVA-fed mice showed an in vitro response to OVA in mesenteric lymph nodes.

The peripheral lymphocyte culture at 7 days after OVA feeding was not successful, having low counts in the PHA wells, however, a second test of peripheral lymphocytes revealed stimulated cells ($P < 0.02$ compared to controls) with a stimulation index of 6.4. Similarly at 14 days after feeding a noticeable response occurred in peripheral lymphocytes with a stimulation index of 4.6 obtained and $P < 0.01$ when the mean cpm was compared to control wells.

3.11 Conclusions and Discussion of Section (b)

Activated T cells can be detected in the mesenteric lymph nodes of CY-treated mice between 6 and 7 days following OVA-feeding and may be an indication of mucosal CMI. However, the response measured by lymphocyte transformation is slight and difficult to reproduce.

Peripheral lymph node cells from normal mice fed OVA also showed some in vitro stimulation in the presence of OVA at 1, 3, 7 and 14 days after antigen feeding. The response at 1 day after OVA feeding was also seen in mesenteric lymph nodes and may illustrate the initiation of responsiveness in the animal. A similar early increase in the proliferative response of mesenteric lymph node cells was previously observed by Silverman et al in mice fed BSA. This response dropped below background level after 2 days and, unlike my results, was not seen in peripheral lymph nodes. (Silverman et al, 1983).

No functional studies were carried out on the responding peripheral lymph node cell population other than determining that the in vitro transformation in response to OVA was T cell dependent. With this in mind it is still interesting to note that the spleens of BDFI

mice fed sheep enthrocytes have been found to contain DTH effector cells (Kagnoff, 1978 b) and, in a separate study, helper T cells for plaque-forming cell responses (MacDonald, 1982). Systemic T cell priming by antigen feeding is no new phenomenon therefore.

The in vitro response of cells from those mice fed antigen and not stimulated with OVA in FCA were extremely low. I was unprepared for this as the assay had been developed using cells from lymph nodes draining footpads injected with OVA in FCA. Under these latter conditions only a small amount of C^{14}-thymidine (0.01 μCi) was required per well in order to detect transformation. The same amount of label used in the later tests may have severely limited detection of the lower responses of mice given OVA orally and even pushed the assay to the limits of its sensitivity. This might account for some of the results which were non-reproducible. The lymphocyte transformations were also prone to variation and this too made data difficult to interpret.

These experiments were being carried out in parallel with another series of experiments designed to investigate in vivo the regulation of systemic responses to orally administered protein antigen by studying the nature of the regulatory stimulus produced as a consequence of antigen feeding. This latter line of research was proving more fruitful than my in vitro studies and eventually when a choice had to be made regarding the final direction of my research within a limited period of time, I chose to follow up the in vivo experiments. These experiments are described in the remainder of the thesis.

CHAPTER 4

ORAL TOLERANCE INDUCTION BY GUT-PROCESSED ANTIGEN

4.1 Introduction

As discussed in the general introduction to this thesis, the intestine has the potential for immunoregulation via physico-chemical modification of ingested antigen: antigen processing. The importance of antigen processing by the gut in the induction of oral tolerance was examined by studying the *in vivo* immunological effect of antigen which had been absorbed across the gut into the serum.

In collaboration with Dr Allan Mowat and Dr Stephan Strobel, a serum transfer protocol was devised. Serum was collected from mice 1 hour after feeding a tolerizing dose of OVA and transferred into syngeneic recipients. The immunological properties of OVA in the transferred serum were then assessed by immunizing the serum recipients with OVA in adjuvant and measuring their subsequent humoral and cell-mediated immunity. A similar protocol was also used to determine the mechanism by which oral tolerance is abrogated by CY. Cyclophosphamide is known to inactivate suppressor cells (Gill and Liew, 1978; Sy *et al*, 1977; Atallah *et al*, 1979), but by virtue of its alkylating action on dividing populations of cells such as enterocytes (Ecknauer and Lohrs, 1976; Sobhon *et al*, 1977), CY may also affect the intestinal processing of OVA such that the antigen does not provide the appropriate immunological stimulus to the host. These hypotheses were tested by injecting serum donors with CY 2 days prior to transfer of their sera and by injecting recipients with CY 2 days prior to serum injection. If gut-processing is affected by CY, then pretreatment of donors might alter the consequent immunological effects of transferred serum. However, if CY abrogates oral tolerance by inactivating suppressor cells then serum recipients given CY should have altered responses to the gut-processed antigen.

4.2 Experimental Design

BALB/c mice were treated according to the following experimental protocol. Groups of 20 donor mice were fed 0.2 ml water (Group A) or else 25 mg OVA in 0.2 ml water (Group B) by intragastric intubation and were bled out from the axillary vein and artery one hour later. The sera collected in each group were pooled and 0.8 ml injected into each of 6 recipients i.p.. Two groups of donors were injected i.p. with 100 mg/kg CY 2 days before feeding water or OVA (Groups C and D) and similarly, two groups of recipients were given CY 2 days prior to injection of serum (Groups E and F). One week after serum transfer, all recipients were immunised by an injection of 100 μg OVA in FCA into the right hind footpad. Recipients were bled from the retro-orbital plexus at 2 and 3 weeks after immunization and their sera assayed for anti-OVA antibodies by haemagglutination. At 3 weeks after immunization the mice were also skintested for DTH by injecting 100 μg OVA in saline into the left footpad and measuring the 24 hour increment in footpad thickness. The basic design of this type of experiment is illustrated in figure 4.

4.3 Systemic Response of Serum Recipients

Recipients of serum from water-fed or OVA-fed donors had identical levels of IgM at 2 weeks and IgG at 3 weeks after immunization (figure 5). In contrast (figure 5), recipients of serum from OVA-fed mice had suppressed DTH responses compared to recipients of serum from mice which were fed water ($P < 0.02$).

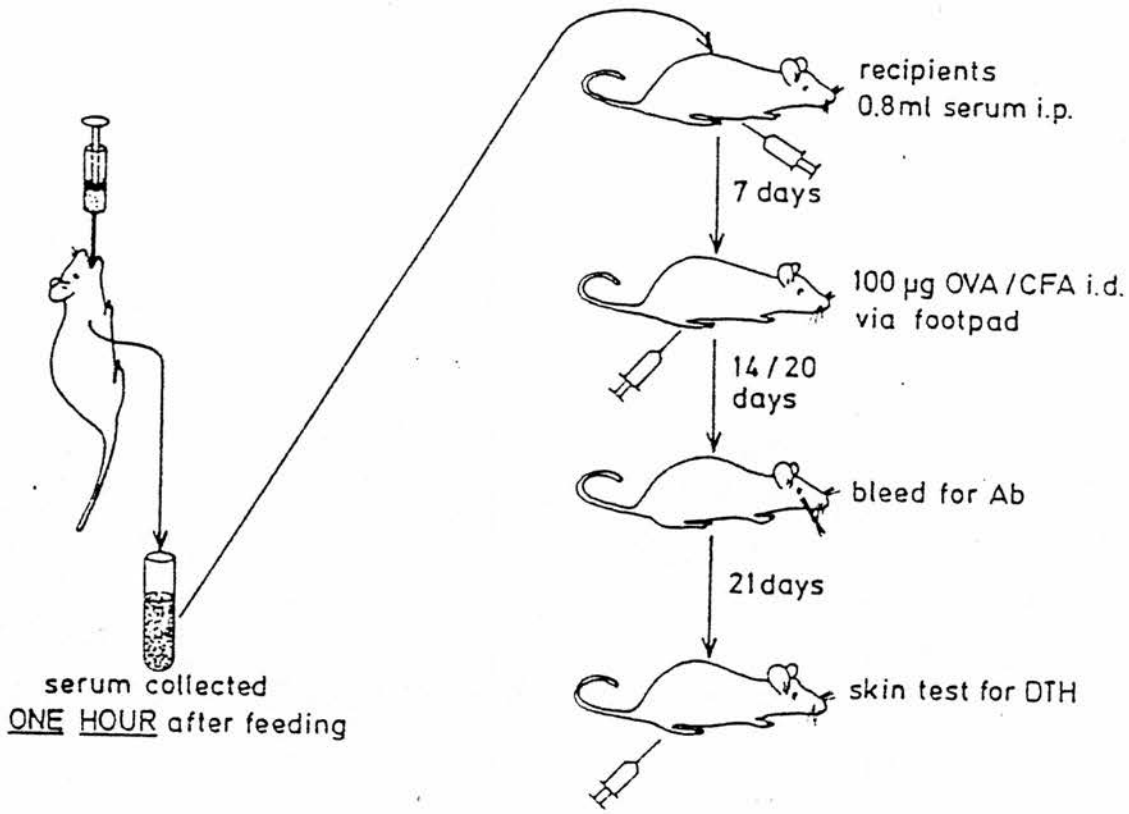


FIGURE 4

Basic design of Serum Transfer Experiments.

Mice were fed 25 mg OVA dissolved in 0.2 ml saline (or H_2O) or 0.2 ml saline (or H_2O) only.

Antibody was measured either by indirect haemagglutination or by ELISA.

DTH was elicited in the footpad by an injection of 100 ug OVA in saline (0.05 ml). The response was measured as the mean increment in footpad thickness 24 hrs later.

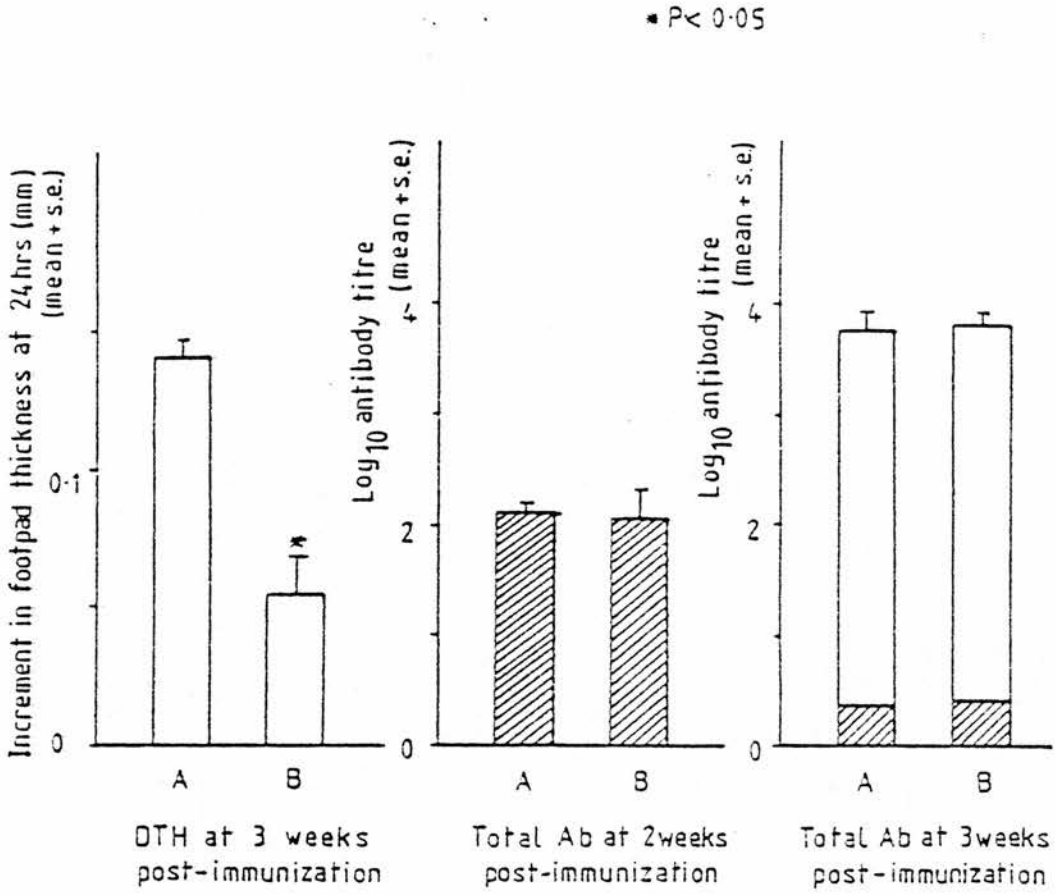


FIGURE 5

Systemic cell-mediated and humoral response of recipients of serum from H_2O fed or OVA fed donors.

Group A; received serum from H_2O fed donors collected 1 hr after feeding

Group B; received serum from OVA fed donors collected 1 hr after feeding

Shading represents mercaptoethanol-sensitive Ab

Antibody was measured by indirect haemagglutination. DTH was measured as footpad swelling 24 hrs after antigen challenge.

Responses were assayed 21 days after OVA immunization.

4.4 Effect of CY Pretreatment of Serum Donors and Recipients

This can be seen in figure 6. Pretreatment of serum donors with CY 2 days before water or OVA feeding (Groups C and D) had no effect on the humoral antibody responses of recipients nor did it affect the suppression of DTH in recipients of serum from CY-treated, water-fed donors (Group C versus Group D, $P < 0.01$).

Serum recipients pre-treated with CY (Groups E and F) showed identical antibody responses to untreated recipients (Groups A and B). However, the suppression of DTH in recipients of serum from OVA-fed donors is abrogated by CY pretreatment of recipients (Groups E and F).

4.5 Antigen-Specificity of Serum-Transferable Tolerance.

The above experiments were performed in BALB/c mice. As I intended using BDFI strain mice throughout my programme of research I repeated the experiments in BDFI mice and included with these a test to determine whether the immunological effects of transferred serum were antigen-specific.

Serum from BDFI mice was collected one hour after feeding OVA or saline as before and transferred into recipients which were subsequently immunized with either 100 µg OVA in FCA or 100 µg human serum albumin (HSA) in FCA. HSA does not cross-react with OVA and was not present in the laboratory mouse diets. Antibody levels in recipients were measured by an ELISA which detected anti-OVA IgG. This test was developed as a means of measuring serum antibody directly as opposed to the indirect haemagglutination assay.

The DTH response of recipients of serum from saline or OVA-fed

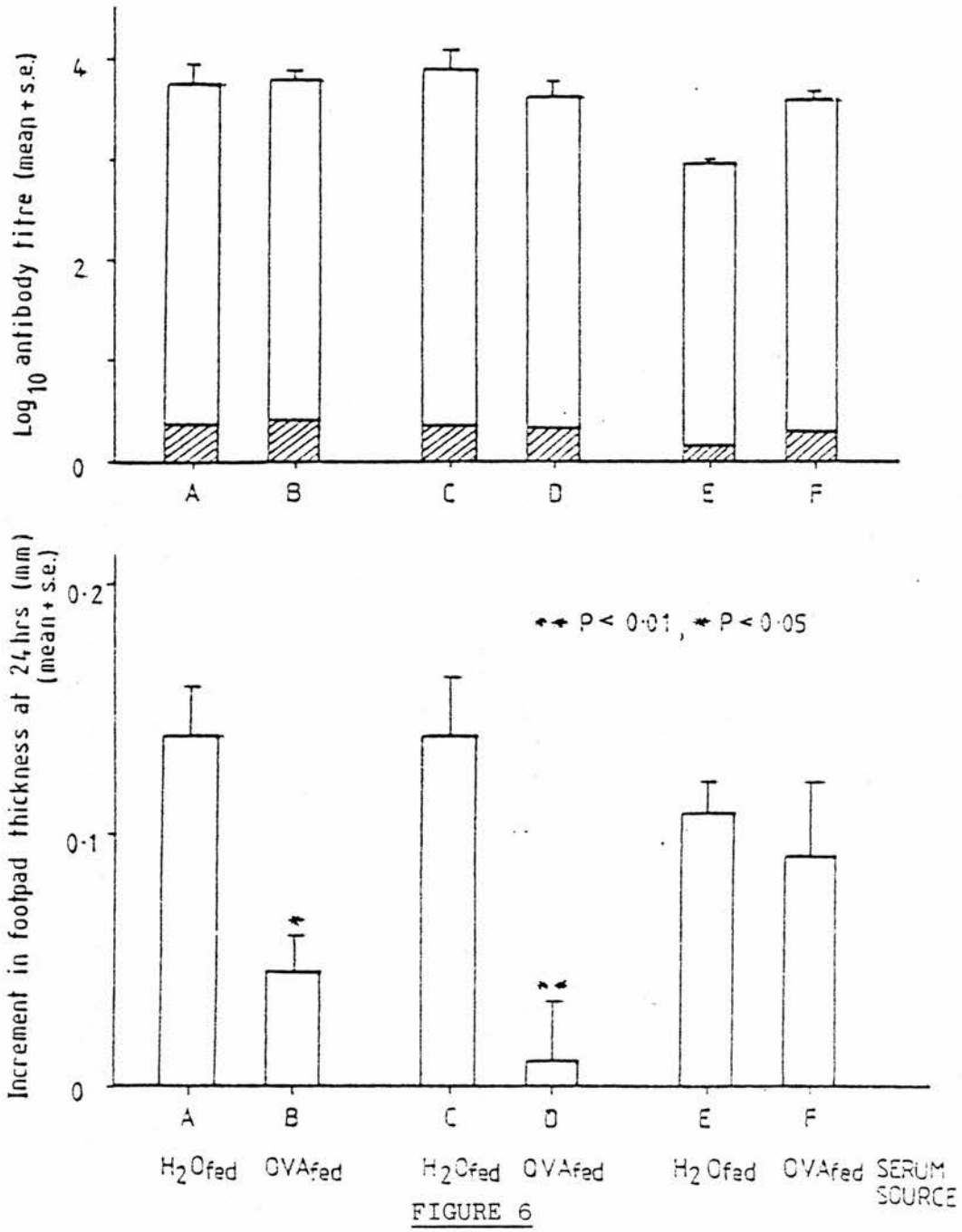


FIGURE 6

Systemic Humoral and Cell-Mediated Responses of Recipients of Serum from H_2O Fed or OVA Fed Donors: Effects of Cyclophosphamide Treatment of Donors and Recipients.

Antibody was measured by indirect haemagglutination. DTH was measured as footpad swelling 24 hrs after antigen challenge.
Responses were assayed 21 days after OVA immunization.
Groups A, C, E received serum from H_2O fed donors
Groups B, D, F, received serum from OVA fed donors
Groups C, D; serum donors treated with CY
Groups E, F; serum recipients treated with CY
Shading represents mercaptoethanol-sensitive Ab

donors 3 weeks after an immunization with OVA or HSA in FCA is shown in figure 7. As seen in BALB/c mice (figure 5) the DTH response to OVA is suppressed by transfer of serum from OVA-fed mice ($p < 0.005$) and not affected by serum from saline-fed mice. The transfer of suppression with serum from OVA-fed mice was therefore confirmed for BDFI mice also. The DTH response to HSA is not suppressed by serum transfer from saline or OVA-fed mice ($P = 0.4$).

Figure 8 confirms that the 2 antigens, OVA and HSA are antigenically distinct. The anti-OVA response of serum recipients, expressed as ELISA readings of optical density at 405 nm, is absent in mice immunized with HSA ($P < 0.01$). Similarly the anti-HSA response of OVA-immunized mice is negative. The lower response of HSA immunized compared with OVA immunized mice indicates that at this dose, HSA does not elicit as high a response as OVA.

These results also demonstrate that the humoral responses of BDFI recipients of serum from OVA-fed syngeneic donors are not suppressed.

4.6 Conclusions

The transfer of tolerance to OVA with serum from OVA-fed BALB/c mice indicates that tolerance of CMI after feeding OVA is induced in these mice by circulating antigen absorbed from the gut. My experiments also confirmed this phenomenon in another strain of mice, BDFI, which shows that the effect is not merely a peculiarity of BALB/c mice. Incorporated into the experiments in BDFI mice was a test of the antigen-specifity of tolerance induced by an injection of serum from OVA-fed mice. Tolerance was OVA-specific. This shows that the induction of tolerance for CMI by serum transfer was a true antigen-specific systemic hyporesponsiveness. The observation of

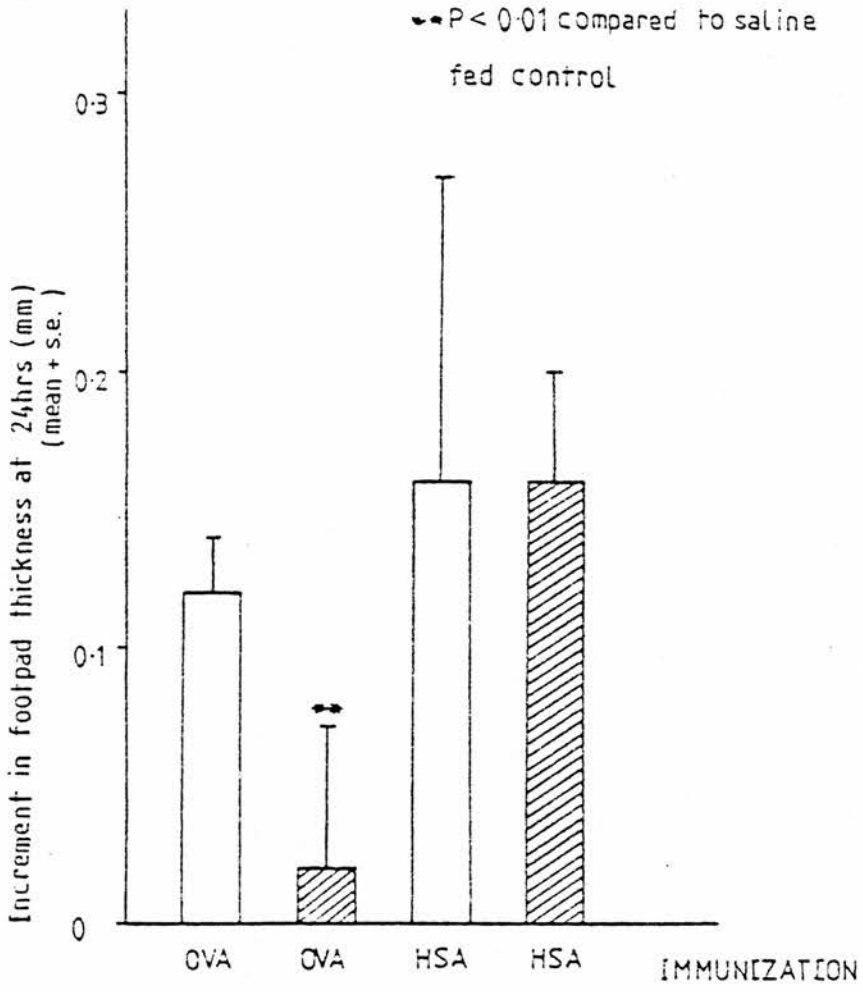


FIGURE 7

Cell-mediated response of recipients of serum from Saline Fed or or OVA Fed donors.

Shaded areas represent those groups of mice injected with serum from OVA fed mice.

Unshaded areas represent groups of mice injected with serum from sal fed donors.

Serum was collected 1 hr after feeding

DTH was measured as footpad swelling 24 hrs after antigen challenge

The response was assayed 21 days after immunization.

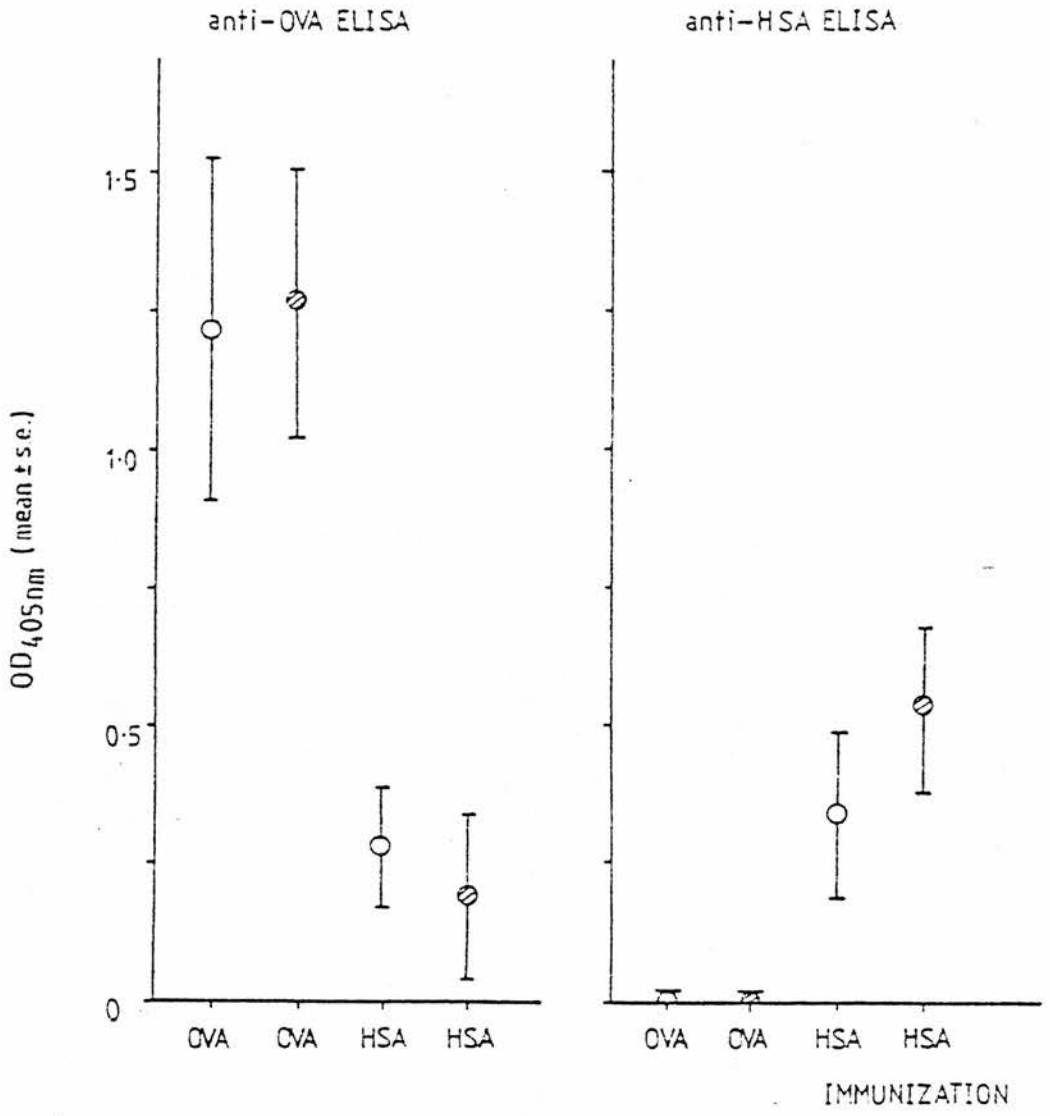


FIGURE 8

Humoral responses of recipients of serum from Saline fed or OVA fed donors.

Shaded areas represent those groups of mice injected with serum from OVA fed mice.

Unshaded areas represent groups of mice injected with serum from sal fed donors.

Serum was collected 1 hr after feeding.

Antibody (IgG) was measured by ELISA (OD 405 nm).

The response was assayed 21 days after immunization.

tolerance for CMI with normal humoral immunity in serum recipients shows that these two limbs of immunity differ in their susceptibility to tolerance induction and may be regulated by separate mechanisms.

Cyclophosphamide injected at a dose of 100 mg/kg does not alter the normal uptake and processing of OVA by the gut since CY-treated serum donors were as efficient as non-treated at processing OVA to induce tolerance in recipients.

The altered response of CY-treated recipients to the tolerogenic stimulus offered by serum from OVA-fed mice indicates the presence of a CY-sensitive mechanism responsible for tolerance induced by OVA feeding.

CHAPTER 5

SYSTEMIC ANTIGEN DOSAGE, FORM AND IMMUNOGENICITY STUDIED IN RELATION TO GUT-DERIVED, CIRCULATING OVALBUMIN

5.1 Introduction

The results of experiments described in Chapter 4, that OVA feeding produced a tolerogen in serum, dictated further research: The suppression of DTH by a serum-borne tolerogen may have been the result of effectively introducing a low dose of OVA into the circulation by feeding, or, the antigen may have been altered to tolerogenic form either by the gut, or by systemic antigen processing once the antigen had reached the circulation.

Estimates of the amount of OVA in serum 1 hour after a single feed of 25 mg OVA have been obtained by co-workers using inhibition of haemagglutination (Mowat, 1982) and ELISA (Strobel, 1983). In agreement with other workers measuring antigen uptakes after feeding, the amount of OVA in serum was found to be extremely variable with a wide range of 0.01% - 1% of ingested dose being detected in the serum. Therefore, in experiments to determine the effect of low doses of OVA on subsequent immunity, mice were injected i.v. with 3 different doses of OVA within this range, that is, 0.1 μg, 1 μg and 10 μg, and were subsequently immunized and challenged.

Alteration in antigen form by intestinal processing was another possible explanation for serum-transferrable tolerance after OVA feeding and so, additional groups of animals were injected with urea-denatured and alkylated OVA (denatured OVA) and also deaggregated ovalbumin over the same range of doses as native OVA and were likewise immunized and challenged with native OVA. Deaggregated proteins are known to be tolerogenic (Lukic *et al*, 1975; Parks and Weigle, 1980 a) and I wanted to test the tolerogenicity of deaggregated OVA in this particular dose range to see whether deaggregated OVA behaved like gut-processed OVA. Similarly if denaturation of proteins reveals

tolerogenic stimuli (Goetzl and Peters, 1972; Takatsu and Ishizaka, 1975), denatured OVA might also behave like a product of gut-processing.

In addition to these i.v. injections of native, denatured and deaggregated OVA, a parallel experiment was set up with these antigens being injected via the intra-peritoneal route. This was done in order to compare the 2 routes of antigen entry; one directly into the circulation, the other via lymphatic drainage, to see whether these effected different immunological signals to the host. Miller et al (1979) had previously demonstrated a difference in response to cell-bound protein antigen administered subcutaneously compared to intravenous injection of the same antigen. In my experiments the serum from mice fed OVA was normally injected i.p. and so it was important to check the effect of route of antigen entry on immunological outcome.

5.2 Experimental Design

Groups of 6 BDFI mice were injected with 0.1, 1 or 10 µg of either native OVA, denatured OVA or deaggregated OVA. In one experiment the injections were given i.v. and in another, i.p.. Each experiment included a group of control mice which were injected with physiological saline either i.v. or i.p. as appropriate. One week later, all mice were footpad-immunized with OVA in FCA and were bled 20 days later in order to assess their humoral antibody response by ELISA. A DTH footpad skintest was carried out on day 21 after immunization and read on day 22.

5.3 Humoral and Cell-Mediated Immunity Following i.v. Injection of OVA

Mice injected i.v. with native or denatured OVA prior to immunization had identical antibody responses to mice injected with saline before immunization. Deaggregated OVA on the other hand, suppressed the anti-OVA response over the entire test range of antigen doses (figure 9).

Similarly, the DTH responses of mice injected i.v. with saline, native OVA or denatured OVA were not significantly different for all three doses of OVA injected whereas deaggregated OVA induced suppression of DTH to native OVA at every dose tested (figure 9).

5.4 Humoral and Cell-Mediated Immunity Following i.p. Injection of OVA

The administration of OVA via the i.p. route produced the same pattern of responsiveness as did i.v. injections (figure 10). That is, deaggregated OVA suppressed both the humoral and cell-mediated responses for each dose used in the experiment whilst native and denatured OVA had no effect on the immune response. These mice behaved like the saline-injected controls and were neither primed nor tolerized by this low dose of antigen.

5.5 Determination of The Role of Systemic Processing of Antigen

To determine the role of the reticulo-endothelial system in rendering OVA tolerogenic subsequent to its entry into the systemic circulation, I performed a "biological filtration" experiment. This

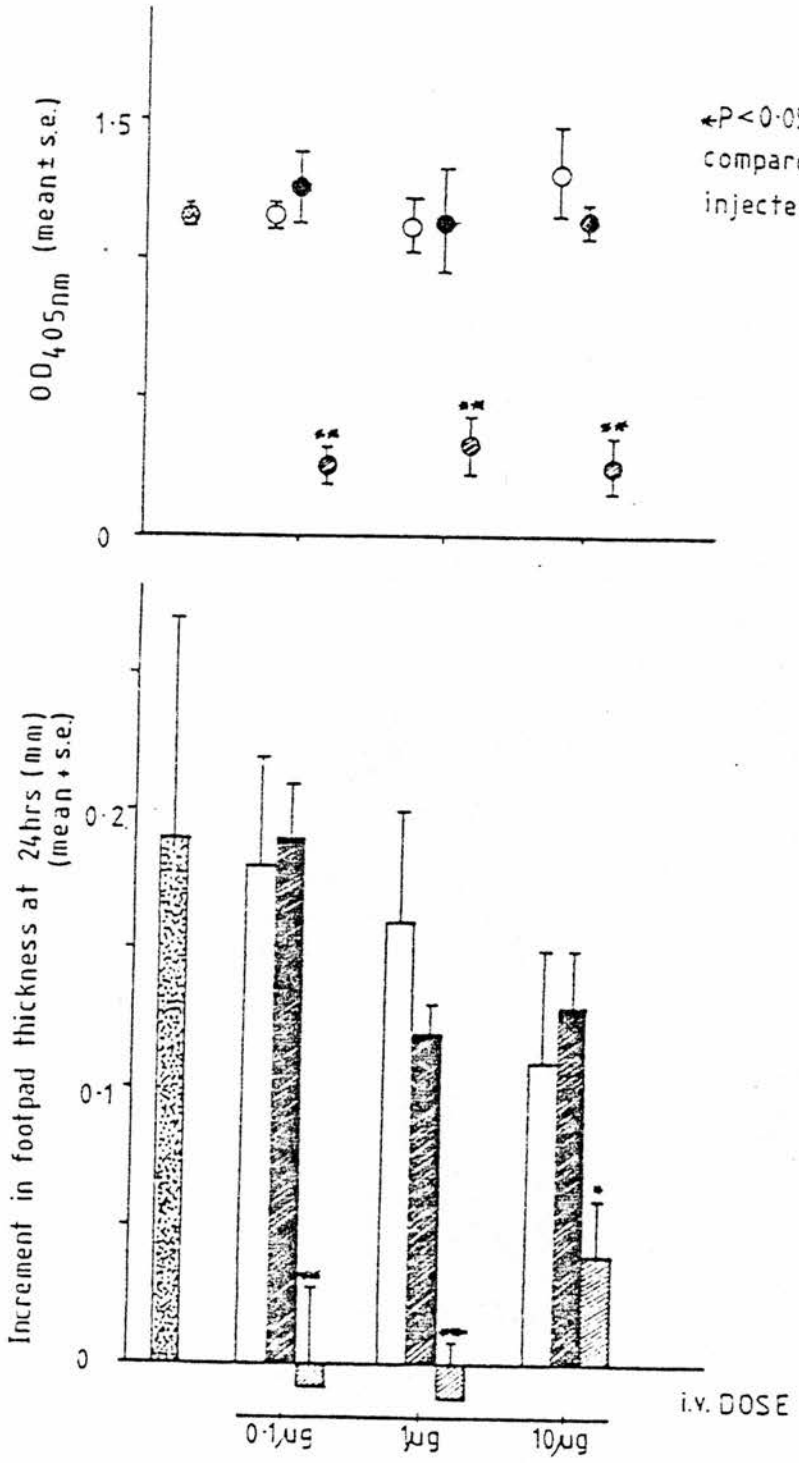


FIGURE 9

Humoral and cell-mediated responses of BDFI mice injected i.v. with native, denatured or deaggregated OVA prior to immunization with native OVA.

Mice were injected i.v. as follows:

Dotted areas; saline, unshaded areas; native OVA, shaded areas den OVA hatched areas; deaggregated OVA.

Antibody (IgG) was measured by ELISA (OD 405 nm). DTH was measured by footpad swelling 24 hrs after antigen challenge.

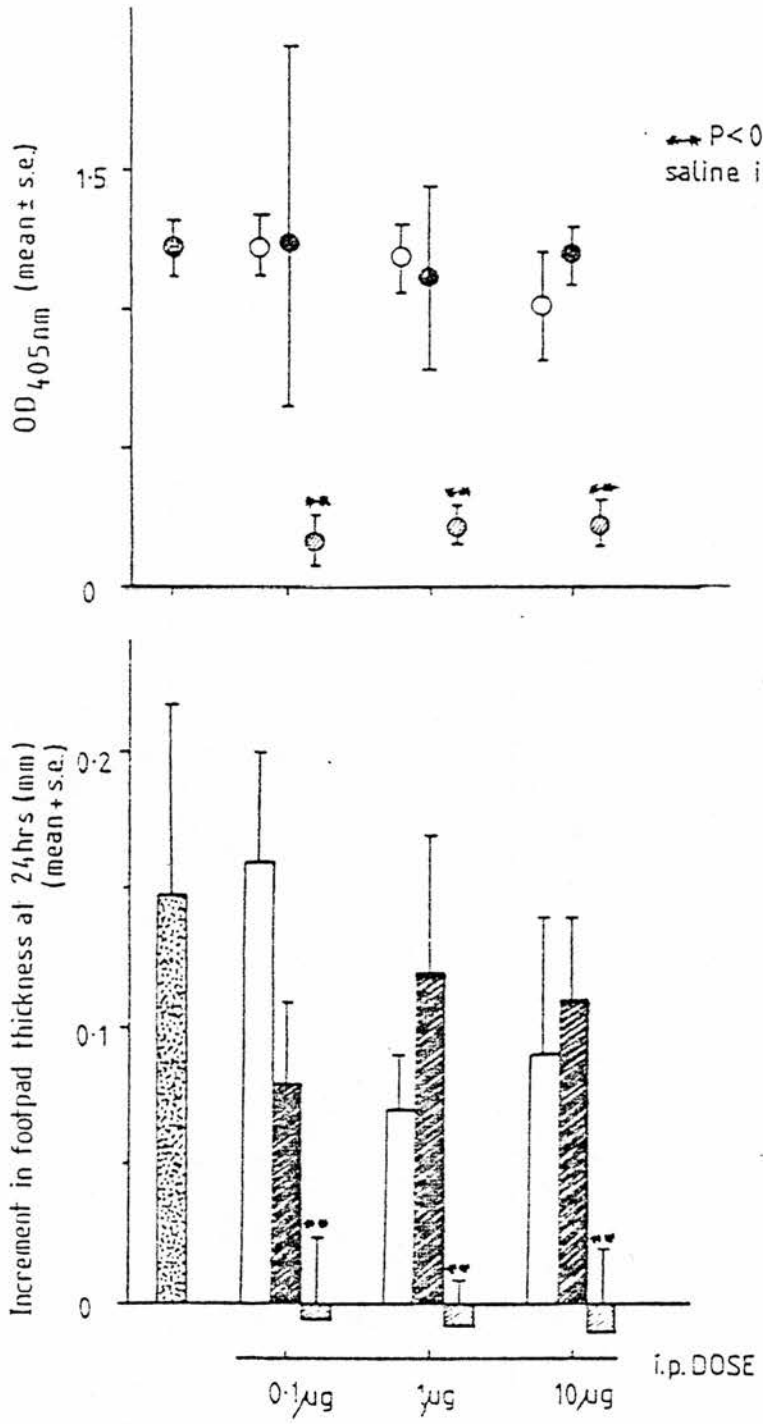


FIGURE 10

Humoral and cell-mediated responses of BDFI mice injected i.p. with native, denatured or deaggregated OVA prior to immunization with native OVA.

Mice were injected i.p. as follows:

Dotted areas; saline, unshaded areas; native OVA, shaded areas; den OVA, hatched areas; deaggregated OVA.

Antibody (IgG) was measured by ELISA (OD 405 nm).

DTH was measured by footpad swelling 24 hrs after antigen challenge.

Responses were assayed 21 days after immunization.

technique had been used by other workers to investigate the importance of antigen processing by macrophages in relation to immunogenicity, (Frei et al, 1965; Lukic et al, 1975)

Mice were injected with antigen which was allowed to circulate for 1 hour and thus be exposed to any systemic antigen processing mechanism. After this time the mice were bled out and their serum containing the "filtered" antigen injected into syngeneic recipients which were then immunized and assessed for antibody and DTH responses.

The i.p. and i.v. routes of injection had produced no difference in immune responses in the previous experiments and all antigen doses tested had produced identical results. Native and denatured OVA had both induced similar responses to native OVA. These antigens were now tested for any difference in their systemic antigen processing. Therefore, donor mice were injected i.p. with saline or a selected dose of 10 µg of either native or denatured OVA and were bled out one hour after injection. The serum of these mice was injected into recipients which were immunized with OVA as described above, bled for serum antibody at 20 days and skintested for DTH on day 21 after immunization.

The responses of serum recipients are shown in figure 11 and reveal that after 1 hour in the systemic circulation, native OVA was not rendered tolerogenic. Denatured OVA was also not altered in its immunogenicity by this procedure and behaved like native OVA producing positive antibody and DTH responses to native OVA which were slightly higher than controls but not significantly different.

5.6 Conclusions

Parenteral administration of OVA via either the i.v. or i.p.

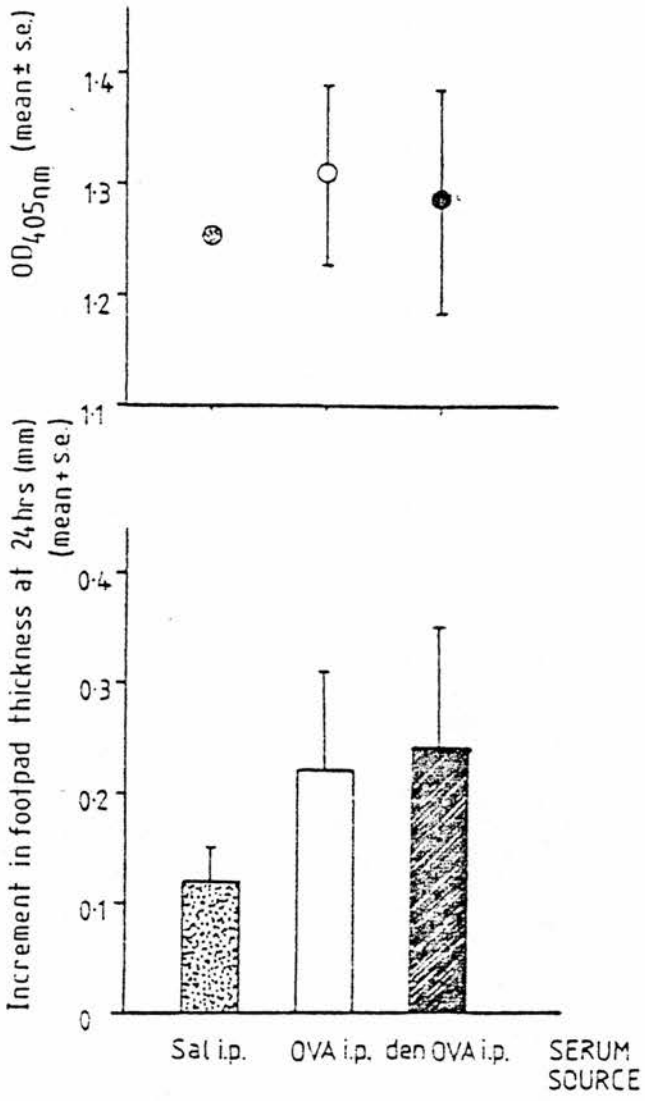


FIGURE 11

Humoral and cell-mediated responses of recipients of serum from donors injected i.p. with saline, native OVA or denatured OVA prior to immunization.

Recipients were injected with serum collected from donors 1 hr after i.p. injections as follows:

Dotted areas; saline, unshaded areas; OVA, shaded areas; den OVA.

Antibody (IgG) was measured by ELISA (OD 405 nm).

DTH was measured by footpad swelling 24 hrs after antigen challenge.

Responses were assayed 21 days after immunization.

route produced identical results: low doses of parenterally injected native or urea-denatured ovalbumin given in a range of amounts previously detected in serum 1 hour after a single feed of 25 mg OVA did not induce tolerance to native OVA. This refutes the hypothesis that tolerance transferred by serum collected 1 hour after an OVA feed is due to the quantity of systemic antigen available.

Urea-denatured OVA did not differ from native in its effect on the immune response and implies that this form of OVA is not a product of gut-processing since the denatured protein was not tolerogenic. Deaggregated OVA produced tolerance, at all doses employed, for both DTH and serum antibody. Whether this reflects gut processing is not clear since circulating gut-derived OVA did not induce tolerance for antibody responses (chapter 4).

OVA which had been in the systemic circulation for 1 hour was not tolerogenic. This excludes the possibility that OVA entering the circulation via the gut is rendered tolerogenic after its absorption and implies that the presence of tolerogenic OVA in serum after feeding is due directly to antigen processing by the gut.

CHAPTER 6

ANTIGENIC CHARACTERISTICS OF GUT-PROCESSED OVALBUMIN

6.1 Introduction

The results of chapters 4 and 5 previously have shown that OVA which is absorbed into the circulation after feeding has been rendered tolerogenic by gut processing and thus presents a suppressive signal to the systemic immune system. Clearly, the characteristics of gut processed antigen are of importance in the determination of a mechanism for oral tolerance and also in the understanding of immune recognition and regulation in general.

As discussed in the introduction to this thesis, different functional classes of lymphocytes possess different antigen recognition elements. B cells recognize certain antigenic determinants by means of surface antibody and evidence exists that suppressor T cells can recognize the same 3-dimensional conformational structures as B cells (Endres and Grey, 1980 (a)).

If gut-processed OVA possesses determinants which B cells recognize and therefore which might also be recognized by suppressor T cells, OVA-specific antibody could be used to probe for them. Further, if these determinants are required for the appropriate tolerogenic stimulus to be presented to the immune system then their removal should influence the immunogenicity of gut-processed OVA.

This hypothesis was tested by collecting serum from mice 1 hour after feeding OVA and incubating the serum with anti-OVA antibody coupled to Sepharose beads. Removal of the beads at the end of the incubation was expected to remove gut-processed OVA bearing B cell determinants. An ELISA technique was used to test serum from OVA-fed mice before and after treatment with Sepharose for the presence of immunoreactive OVA and the various binding phases of this ELISA are described schematically in figure 12. The immunogenicity of absorbed

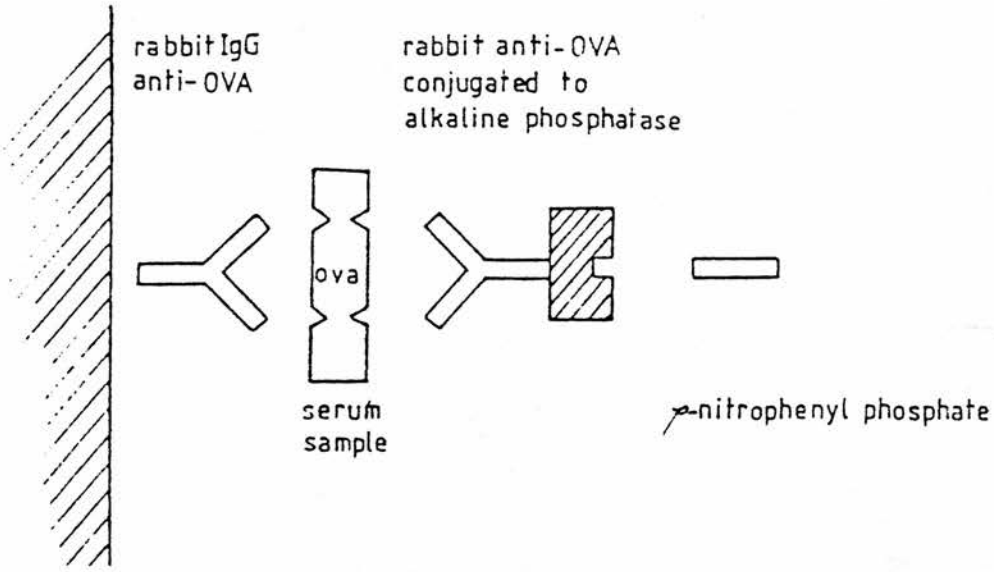


FIGURE 12

Schematic diagram of ELISA for OVA detection

Plates are coated with rabbit IgG anti-OVA.

Serum is added (containing gut-processed OVA).

OVA in serum is detected with rabbit anti-OVA coupled to alkaline phosphatase activated by p-nitrophenyl phosphate.

serum was then tested in vivo to determine the functional significance of antibody-binding determinants in the induction of tolerance by gut-processed OVA.

The nature of helper T cell recognition of gut-processed OVA was not addressed by the experiments described above. This is a more difficult problem to analyse since the means of T cell recognition of antigen is largely unknown. However, in an attempt to investigate whether gut-processed OVA possessed determinants which T cells recognise, gut-processed OVA was added to cultures of lymphocytes from mice immunized with OVA in Freund's complete adjuvant in order to test whether OVA-primed helper T cells could be stimulated by the serum-borne OVA to transform in vitro.

6.2 Use of Antibody-Coupled Sepharose as an Immunoadsorbent

Hyperimmune rabbit anti-OVA anti-serum was used as a source of OVA-specific antibodies. Antibody was coupled to beads of Sepharose 4B, referred to as S4B-anti-OVA, and (Chapter 2, Section c) 200 µl of S4B-anti-OVA could bind 5 µg OVA. A minimum estimate of 200 ng OVA per ml of serum taken 1 hour after feeding 25 mg OVA was adopted based on the results of Dr Strobel (personal communication 1983), and so 200 µl of S4B-anti-OVA added per 5 ml of serum would theoretically contain more than enough antibody to remove an estimated 1 µg of OVA.

6.3 Treatment of Serum to Remove Gut-Processed OVA Bearing B Cell Determinants

Groups of mice were fed either 0.2 ml saline i.g. or 25 mg OVA and were bled out 1 hour later. A portion of each serum pool was incubated with S4B-anti-OVA overnight at room temperature on a

revolving table. One aliquot of serum from OVA-fed mice was incubated twice using fresh S4B-anti-OVA for the second incubation. This was done to ensure the removal of all B cell-recognisable OVA from the serum. Serum from saline-fed mice was also subjected to a double incubation with S4B-anti-OVA. At the end of the incubation period, the Sepharose was allowed to settle and the supernatant sera were pipetted off. From each group, sufficient serum was kept aside and tested in an ELISA for its concentration of OVA. The values obtained were then compared with known OVA concentrations on a standard curve.

6.4 Ovalbumin Concentration in Serum Before and After Incubation With S4B-Anti-OVA

Table 6 lists the amount of OVA detected by ELISA in aliquots of pooled sera from 15 mice. The standard curve for this assay is given in figure 13 and shows a linear relationship between optical density at 405 nm (OD 405 nm) and $\log_{10}$ OVA concentration for values betweeen 1 µg and 31.6 ng per ml ($r = 0.99$). Readings of less than 31.6 ng per ml are therefore beyond the limits of accurate detection of the assay. Serum from OVA-fed mice produced an ELISA reading within the linear range giving a concentration of 38.0 ng per ml.

Serum from saline-fed mice which had been absorbed twice with S4B-anti-OVA had an approximate reading of 7.59 ng/ml and since this serum contained no OVA, all readings of 7.59 ng/ml or less are considered as "background". therefore, the sera from saline-fed mice and from OVA-fed mice after either 1 or 2 incubations with S4B-anti-OVA can be said to contain no immunoreactive OVA.

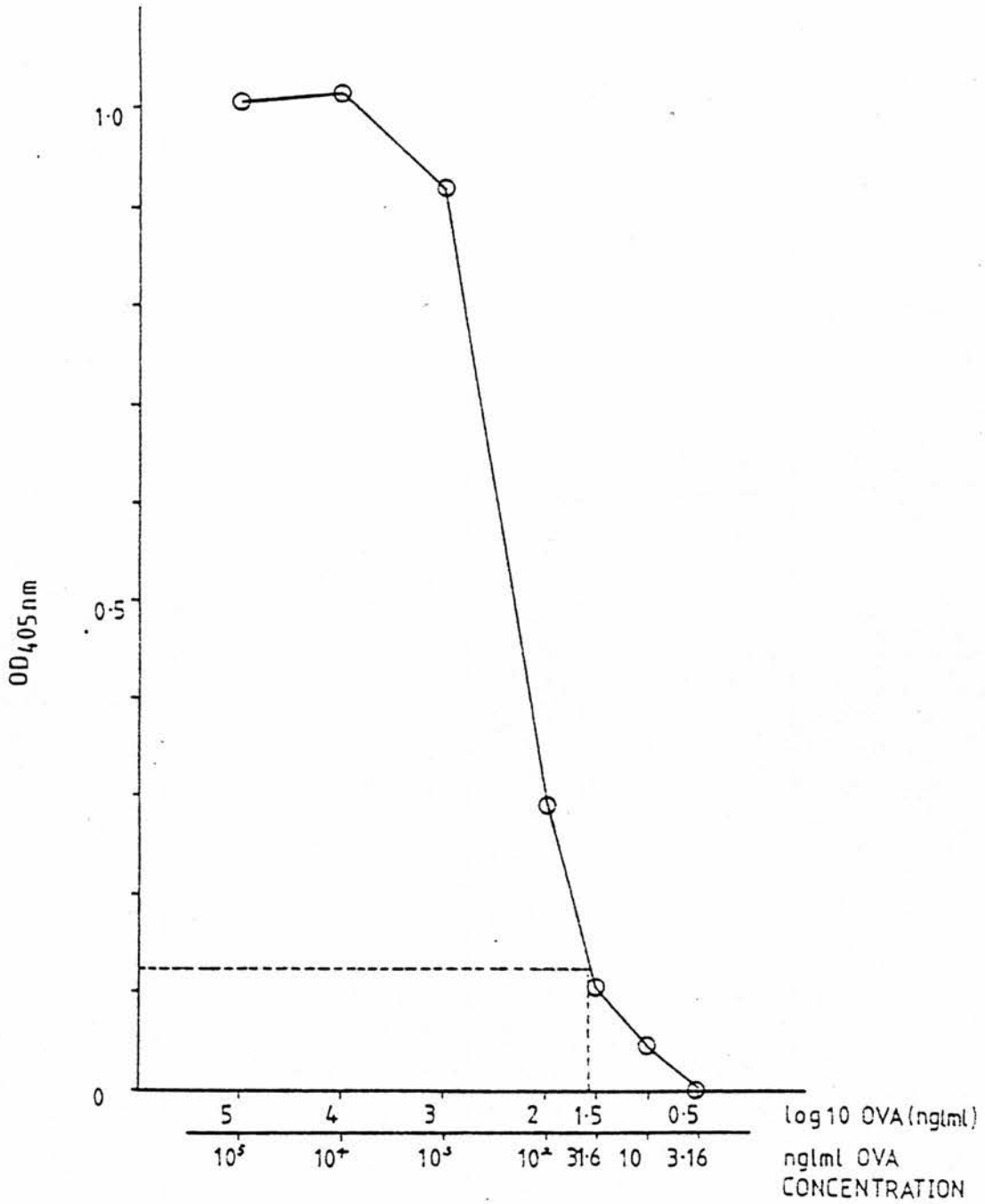


FIGURE 13

Standard curve of ELISA for OVA concentration.

The reading for serum from OVA fed mice is indicated by broken lines.

The concentration of OVA is found by taking the antilog of 1.57;

38 ng/ml

	SERUM SOURCE				
	Sal-fed	OVA-fed	OVA-fed abs x 1	OVA-fed abs x 2	Sal-fed abs x 2
OVA ng/ml (pooled sera, n = 15)	5.01	38.0	6.3	5.01	7.59

Lower limit of assay at 31.6 ng

TABLE 6

OVA (By ELISA) in serum of BDFI mice after saline or OVA feeding ± incubation with S4B-Anti-OVA

Serum was collected from BDFI mice 1 hr after Sal or OVA feeding and treated with S4B-anti-OVA. "abs" refers to the number of incubations with S4B-anti-OVA.

6.5 Immunogenicity in vivo of Serum Absorbed with S4B-Anti-OVA

Groups of recipient mice were injected i.p. with 0.8 ml of the serum from saline or OVA-fed mice which was prepared in section 6.3 above, and assayed for OVA concentration in section 6.4. One week after serum injection, the mice were immunized with OVA in FCA as usual and were bled and DTH skintested on days 20 and 21 respectively. The systemic humoral and cell-mediated responses of these mice are shown in figure 14.

Serum from OVA-fed mice suppressed the DTH response of recipients compared to recipients of serum from saline-fed mice which had strongly positive DTH ($P < 0.05$). Serum from OVA-fed mice which was absorbed either once or twice with S4B-anti-OVA did not suppress the DTH response to OVA when compared either with the response of recipients of serum from saline-fed mice, or with that of recipients of twice-absorbed serum from saline-fed mice.

The humoral antibody response of recipients of serum from OVA-fed mice was significantly lower than the controls which were injected with serum from saline-fed mice ($P < 0.05$). However, there was no difference in the response of recipients of once or twice-absorbed serum from OVA-fed mice compared to their controls which had received twice-absorbed serum from saline-fed mice.

6.6 Immunogenicity in vitro of Gut-Processed OVA

Groups of BDFI mice were fed either 0.2 ml saline or 25 mg OVA i.g.. They were bled out 1 hour after feeding, the sera were pooled within groups and the concentration of OVA assayed by ELISA. The standard curve for this assay is shown in figure 15 and the relationships between OD 405 nm and $\log_{10}$ OVA concentration was linear within the range from 100 μg per ml to 31.6 ng per ml OVA in serum (r

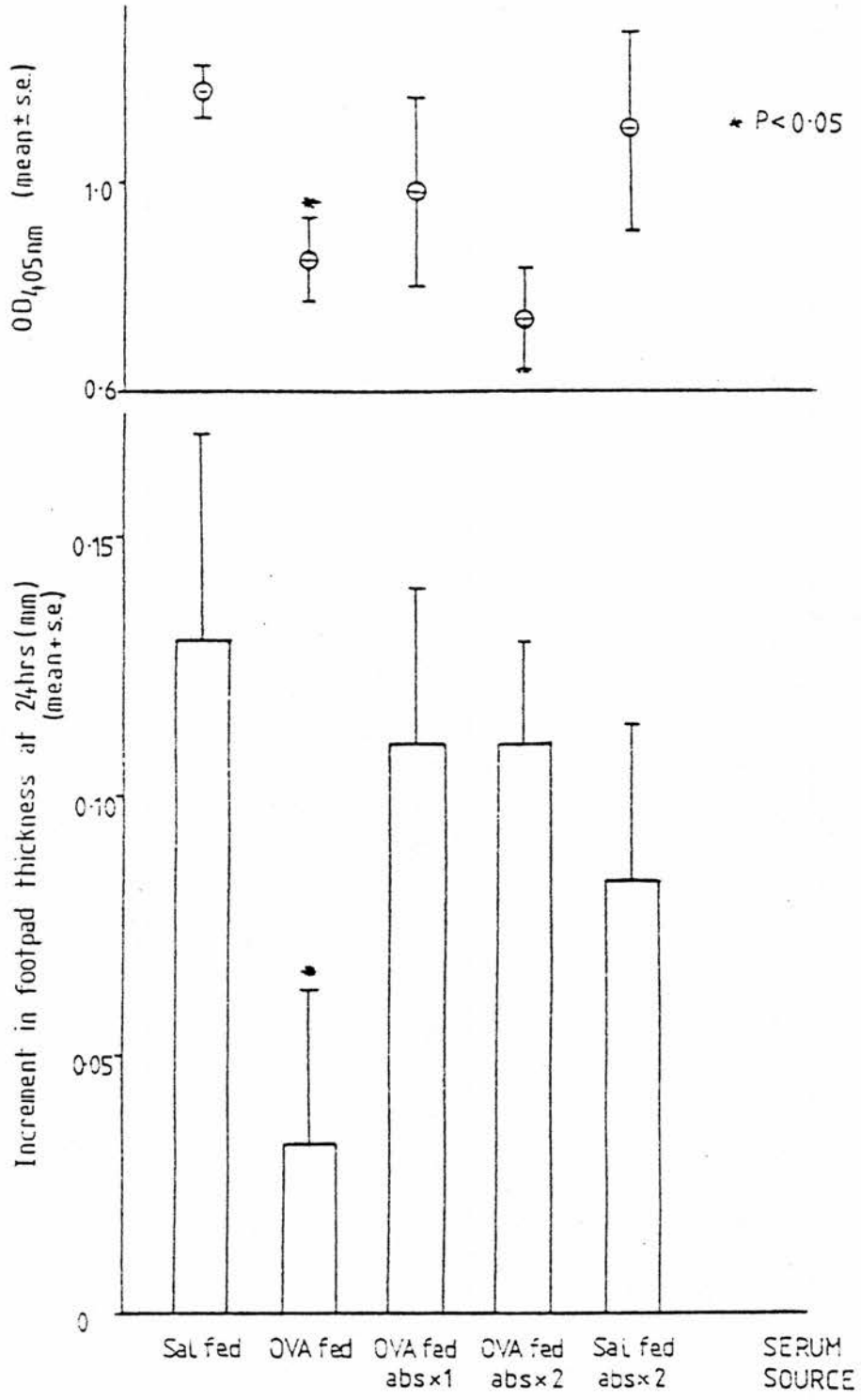


FIGURE 14

Systemic humoral and cell-mediated responses of recipients of serum from saline or OVA fed mice: Effects of incubating serum with anti-OVA.

Serum was collected from mice 1 hr after feeding.
Antibody (IgG) was measured by ELISA.
DTH was measured by footpad swelling 24 hrs after antigen challenge.
Responses were assayed 21 days after immunization.
Recipients were injected with serum from donors treated as shown and were immunized 1 week later.

The term "abs" (absorbed, refers to sera which were incubated with the immunoadsorbent S4B-anti-OVA).

= 0.99). The reading obtained for serum from OVA-fed mice falls in this range as shown on the graph and corresponds to a value of 40 ng/ml.

As a source of primed cells for culture with gut-processed OVA, BDFI mice were footpad-immunized with 100 μg OVA in FCA. Three weeks later they were killed and cell suspensions made from the draining lymph nodes.

The optimum antigen dose for T cell-dependent antigen-driven blast transformation was 1.25 mg/ml OVA, that is, 250 μg OVA per well. The amount of OVA present in the serum after feeding was only 40 ng per ml. Therefore, the culture plate included wells containing; cells only, cells + PHA as a positive control, cells + 250 μg OVA, cells + 10 μl serum from OVA-fed mice (this contained 40 ng gut-processed OVA per ml), and cells + 10 μl of normal mouse serum containing native OVA at a concentration of 40 ng per ml in order to compare any transformation with gut-processed OVA to an appropriate dose of native OVA. Cells + 10 μl normal mouse serum were also included to isolate the effect of homologous serum proteins on cells in culture.

In an effort to increase the dose of gut-processed OVA presented to cells in vitro, serum from OVA-fed mice was concentrated using ultrafiltration membranes and the effective OVA dose calculated by means of a concentration factor as shown in figure 16. Increasing volumes of concentrated serum were added to cultures and cultures with corresponding amounts of concentrated normal mouse serum were included as controls. The results are summarised in table 7.

The response of OVA-primed cells to a 250 μg dose of OVA was positive as expected with a mean cpm of 2953 $\pm$ 1235 ($P < 0.02$ compared to unstimulated cells). There was no in vitro response to

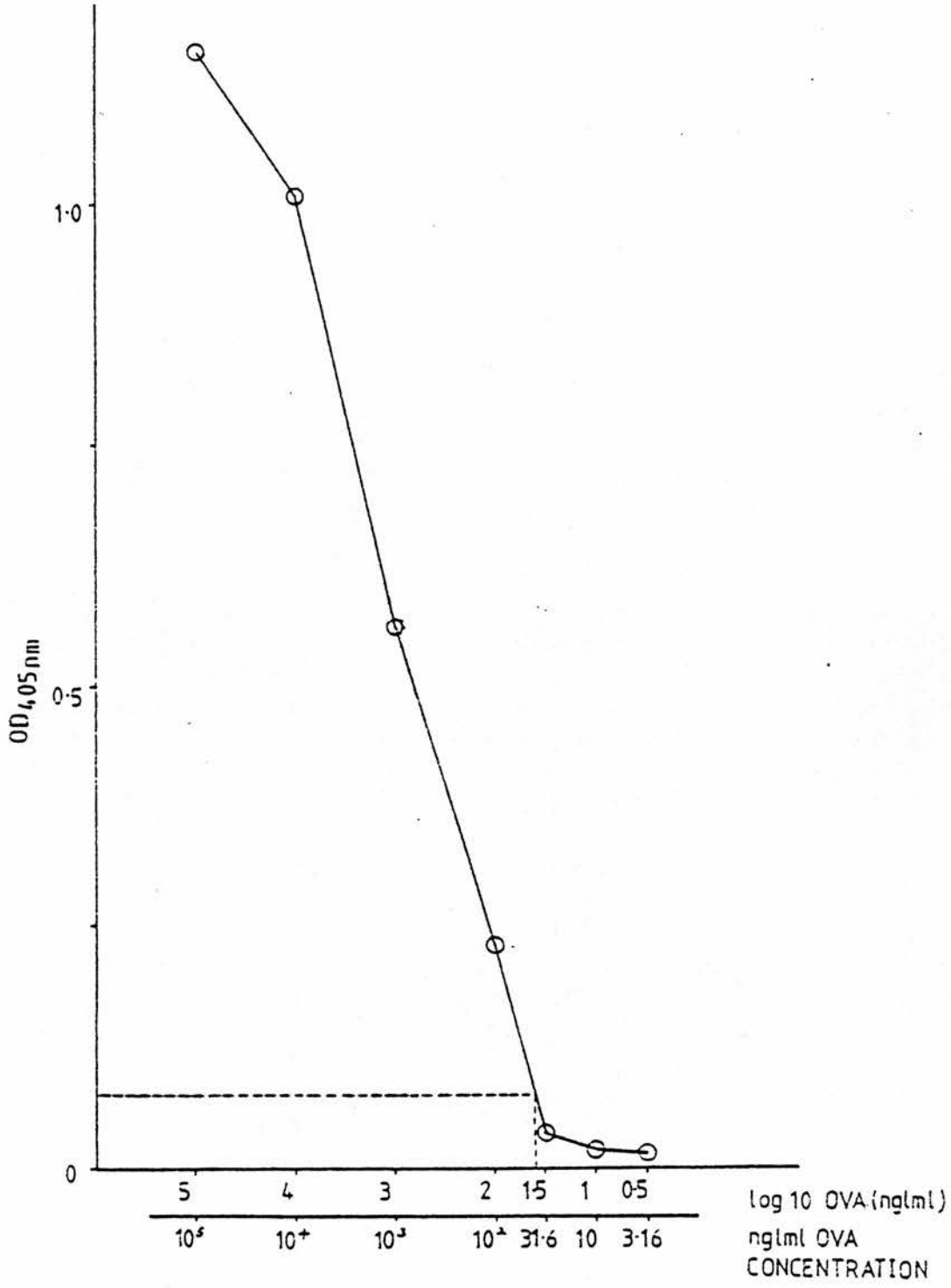


FIGURE 15

Standard curve of ELISA for OVA concentration.

The reading for serum from OVA fed mice is indicated by broken lines.

The concentration of OVA is found by taking the antilog of 1.6; 40 ng/ml.

After Ultrafiltration

Volume of concentrate = 0.9 ml

Volume removed = 2.3

Concentration factor = $\frac{(2.3 + 0.9)}{0.9}$

= 3.6

Amount of OVA in neat serum
(measured by ELISA) = 40 ng/ml

Amount of OVA in concentrated serum

= 40 x 3.6 ng/ml

= 144 ng/ml

Therefore,

10 µl concentrated serum contains 1.44 ng OVA

25 µl concentrated serum contains 3.6 ng OVA

50 µl concentrated serum contains 7.2 ng OVA

After ultrafiltration of normal mouse serum,

Volume of concentrate = 0.7 ml

Volume removed = 1.3 ml

Concentration factor = $\frac{(1.3 + 0.7)}{0.7}$

= 2.9

FIGURE 16

Calculation to estimate the amount of OVA in concentrated serum.

Serum was collected from mice 1 hour after OVA feeding and concentrated by ultrafiltration

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Added to Cultures	Mean cpm ± s.e.	Stimulation Index	P Value
PHA	2313 ± 526	6.7	0.025
250 µg OVA	2953 ± 1235	8.6	0.02
-	345 ± 327	1	-
0.4 ng "processed" OVA	34 ± 8	0.1	N. S.
0.4 ng OVA in NMS	52 ± 47	0.15	N. S.
10 µl NMS	20 ± 15	0.06	N. S.
1.44 ng "processed" OVA	7 ± 8	0.02	N. S.
10 µl concentrated NMS	64 ± 63	0.18	
3.6 ng "processed" OVA	16 ± 17	0.05	N. S.
25 µl concentrated NMS	30 ± 21	0.09	
7.2 µl "processed" OVA	8 ± 4	0.02	N. S.
50 µl concentrated NMS	8 ± 1	0.009	

TABLE 7

Responses of OVA-primed Lymphocytes to Gut-Processed OVA in vitro

Normal mouse serum or serum containing gut-processed OVA was added to cells obtained from BDFI mice 21 days after immunization with OVA.

serum from OVA-fed mice which contained 0.4 ng OVA, nor to 0.4 ng native OVA dissolved in normal mouse serum ($P < 0.2$ in each case). Normal mouse serum itself induced no *in vitro* response.

Sera which were concentrated before being added to cultures in increasing amount produced no *in vitro* response and the response of cells given concentrated normal mouse serum was no different from cells given concentrated OVA-containing serum.

6.7 Conclusions

Suppression of DTH in recipients of serum from OVA-fed mice is abrogated if the serum is treated with the immunoadsorbent S4B-anti-OVA prior to transfer.

An ELISA which detects OVA by its binding to anti-OVA antibody, confirmed the presence of OVA in the serum after feeding and demonstrated the removal of immunoreactive OVA from serum by S4B-anti-OVA.

Taken together, these observations suggest that the tolerogen present in mouse serum 1 hour after feeding is OVA and that it possesses 3-dimensional conformation for B cell recognition via antibody. The humoral antibody response in recipients of serum from OVA-fed mice was lower than controls in this experiment ($P < 0.05$), and might indicate that a critical dose of antigen is required for the suppression of antibody responses by serum tolerogen.

Serum from OVA-fed mice was unable to stimulate OVA-primed lymph node cells to proliferate in a T-dependent lymphocyte transformation test. This may well have been due to the low dose of OVA present in the cultures as even when the serum was highly concentrated, the maximum antigen dose reached was only 7.2 ng. It would therefore be

unwise to conclude from these results that T helper cells do not recognise determinants on OVA which has been absorbed into the circulation after feeding.

CHAPTER 7

SIZE, DISTRIBUTION AND EFFECT OF OVA FRAGMENTS GENERATED BY GUT-PROCESSING

7.1 Introduction

The previous chapter demonstrated that the tolerogenicity of whole serum collected one hour after an OVA feed is associated with possession of determinants for anti-OVA antibody. However, there still remained many unanswered questions pertaining to this serum-borne tolerogen.

Details of the distribution and molecular size of the tolerogenic moiety in serum would be of interest: Is the tolerogen a single structure or is there a range of various fragments of OVA of differing size and immunogenicity present in the serum? Together with the antigen which is detected in serum by antibody, are there also fragments of OVA which are not capable of being found by antibody but can be recognized by T cells? Do small pieces of OVA molecules bind to serum (self) proteins and thus present as large molecules bearing OVA determinants or as antigen in the context of self determinants? It is feasible that while the dominating effect of processed OVA in the whole serum is to suppress cell-mediated immunity, the effect of these hypothetical OVA fragments in isolation may be to induce quite different immune responses.

This theory is not unprecedented as other workers have shown previously that the immune response to a particular antigen can be altered by changing the structure of the antigen (Chapter 1, section 1.3). Of particular interest is the work of Michael et al (1981) where peptic digestion of BSA in vitro produced fragments of varying molecular weights and antigenicity, the higher molecular weight fragments which were produced could be bound by anti-BSA antibody, and a low molecular weight fragment was also produced which did not bind to antibody but which induced suppressor T cells in mice. This

suppressor fragment could exert a suppressive effect on B cells when conjugated to mouse gamma globulin.

If a similar mixture of OVA fragments is generated by gut-processing, the isolation and both structural and functional investigation of these fragments has potential for gaining insight into the immuno-regulatory mechanisms induced by feeding OVA and also for gaining information in general about mechanisms of antigen recognition by cells of the immune system.

In the first steps towards this end, a series of experiments was devised where serum containing gut-processed antigen was fractionated by gel filtration to separate proteins on the basis of molecular weight. High, medium and low molecular weight fractions were obtained and assayed for total protein content, the presence of OVA with determinants for anti-OVA and any effect in vivo on the immune system. Such an effect of a fraction could therefore be traced to molecules within a known molecular weight range and to whether or not the effect was synonymous with the presence of determinants recognized by antibody.

7.2 Fractionation of Serum Proteins By Gel Filtration

Mice were fed either 0.2 ml saline or 25 mg OVA and were bled out 1 hour after feeding. The pooled sera from each group were then fractionated on a Sephacryl column in order to separate the serum proteins on the basis of molecular weight. Details of the column, its calibration and the fractionation procedure are given in chapter 2 (Materials and Methods, Section (c)).

Samples were collected from the column in 3 ml volumes and pooled into 3 major fractions: A high molecular weight fraction, FI,

containing all serum proteins with molecular weights greater than 100,000 and therefore including the serum proteins α_2 macroglobulin, immunoglobulin A and large lipoproteins; a "medium" molecular weight fraction, FII, which was selected to contain proteins within approximately the same molecular weight range as native OVA; and a low molecular weight fraction, FI, which contained any proteins of molecular weight less than that of native OVA down to the total elution point of the column where very small molecules of 1 or 2 amino acids are eluted. Figure 17 shows the optical density profiles at 206 nm of normal mouse serum proteins with the position of OVA (molecular weight = 43,500) and glycyl-tyrosine (molecular weight = 238) superimposed. The positions of fraction boundaries are also indicated.

Fractions I, II, and III thus obtained were concentrated back to the original serum pool volume by ultrafiltration and were dialysed to remove sodium azide which was present in the column gel buffer.

7.3 Protein and OVA Content of Fractions

Aliquots of serum fractions were assayed for total protein concentration using the method of Lowry and also for immunoreactive OVA by ELISA. The results of these tests are summarised in table 8 together with values obtained for whole normal and OVA-fed mouse serum.

The total protein content of serum from both saline and OVA-fed mice was located in fractions I and II. No protein was detected in the low molecular weight fraction from either saline or OVA-fed mice.

OVA was detected by antibody (in an ELISA) in whole serum of OVA-fed mice and in fraction II of this serum. OVA was not detected

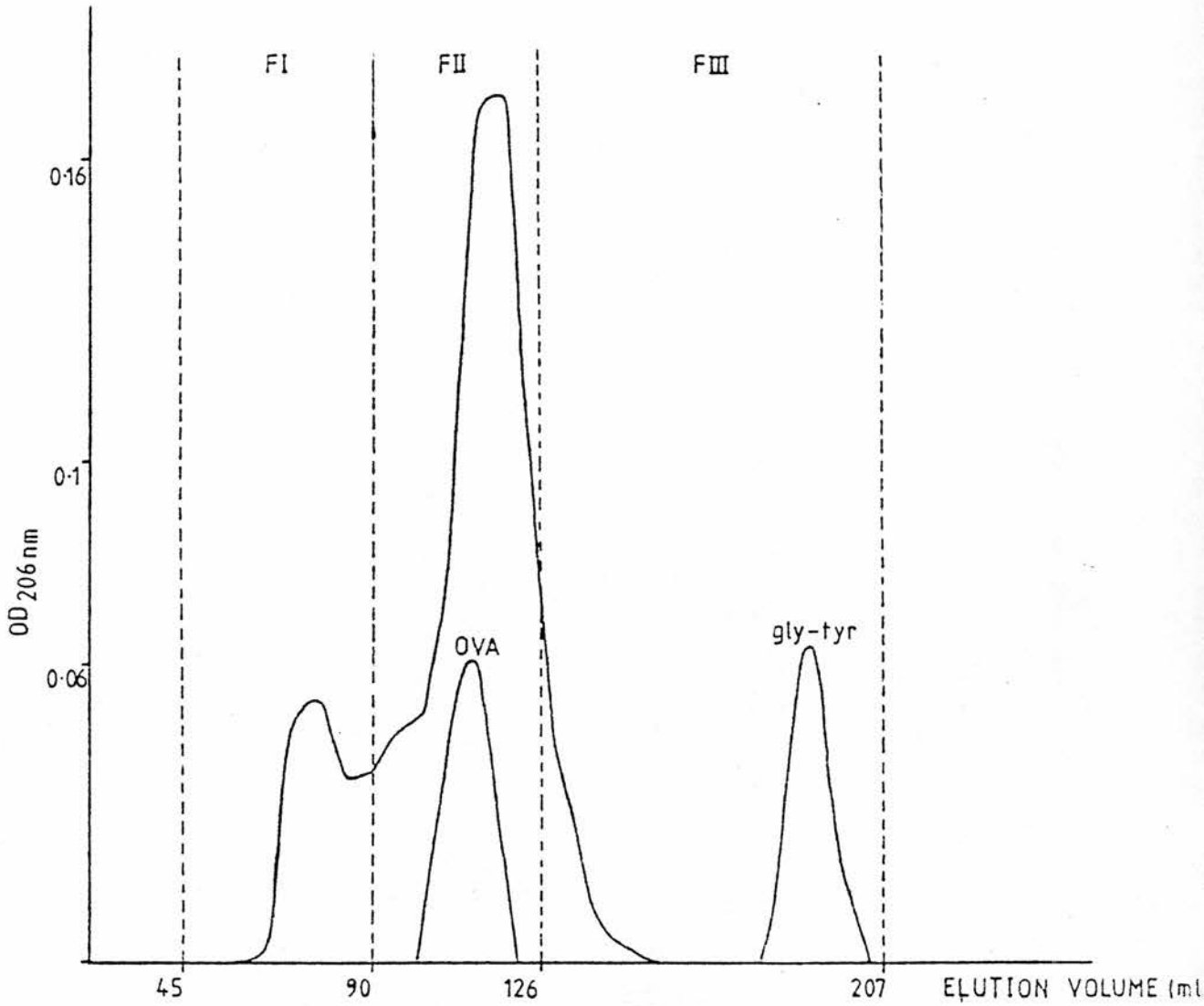

FIGURE 17

Elution profiles of normal mouse serum, OVA and glycyl-tyrosine.

These proteins were eluted from a gel filtration column on the basis of molecular weight. Fractions of serum were selected for transfer experiments. Fraction boundaries are indicated by broken lines.

gly-tyr = glycyl tyrosine.

	NMS Whole	SFS FI	SFS FII	SFS FIII	OFS Whole	OFS FI	OFS FII	OFS FIII
Protein (mg/ml)	32	6.4	25	neg	52.6	7.7	26.3	neg
OVA (ng/ml)	neg	*	neg	neg	38	neg	35	neg

TABLE 8

Estimates of Protein and OVA content in serum and serum fractions of Saline (SFS) and OVA fed (OFS) mice.

Serum was collected from BDFI mice 1 hr after either Sal or OVA feeding.

Serum was fractionated by gel filtration on S200 Sephacryl.

Protein was estimated by Lowry, OVA by ELISA.

* Sample was contaminated before ELISA could be carried out.

in either FI or FIII from OVA-fed mice or in any of the samples from saline-fed mice.

7.4 Immunogenicity of Serum Fractions in vivo

The serum fractions obtained in 7.2 above were tested in vivo for any effect on immunoregulation within the usual immunization regimen. That is, 0.8 ml serum fraction was injected i.p. into recipients which were immunized with 100 µg OVA/FCA one week later and then bled and DTH-skintested 3 weeks after immunization.

In this experiment, mice were also bled just prior to immunization in order to determine whether a humoral response had been mounted to the ingested, absorbed antigen in the serum fraction. Products of the host response to orally administered antigen have been previously postulated, as being responsible for oral tolerance induction (André et al, 1975).

The humoral antibody response in serum fraction recipients immediately before, and 3 weeks after immunization are shown in figure 18. The response of mice injected i.p. with 0.8 ml saline 1 week prior to OVA immunization is included as an illustration of a typical antibody response. Pre-immunization antibody levels were negligible and showed that host antibody was not produced by an injection of serum fraction per se. Three weeks after immunization, the antibody response in all groups was positive and no significant difference was observed when recipients of "OVA-fed" fractions were compared to their appropriate "saline-fed" controls.

The DTH response in recipients of serum fractions from saline-fed mice were positive, as illustrated in figure 19 and a typical positive DTH response was seen in the saline-injected control group. The DTH

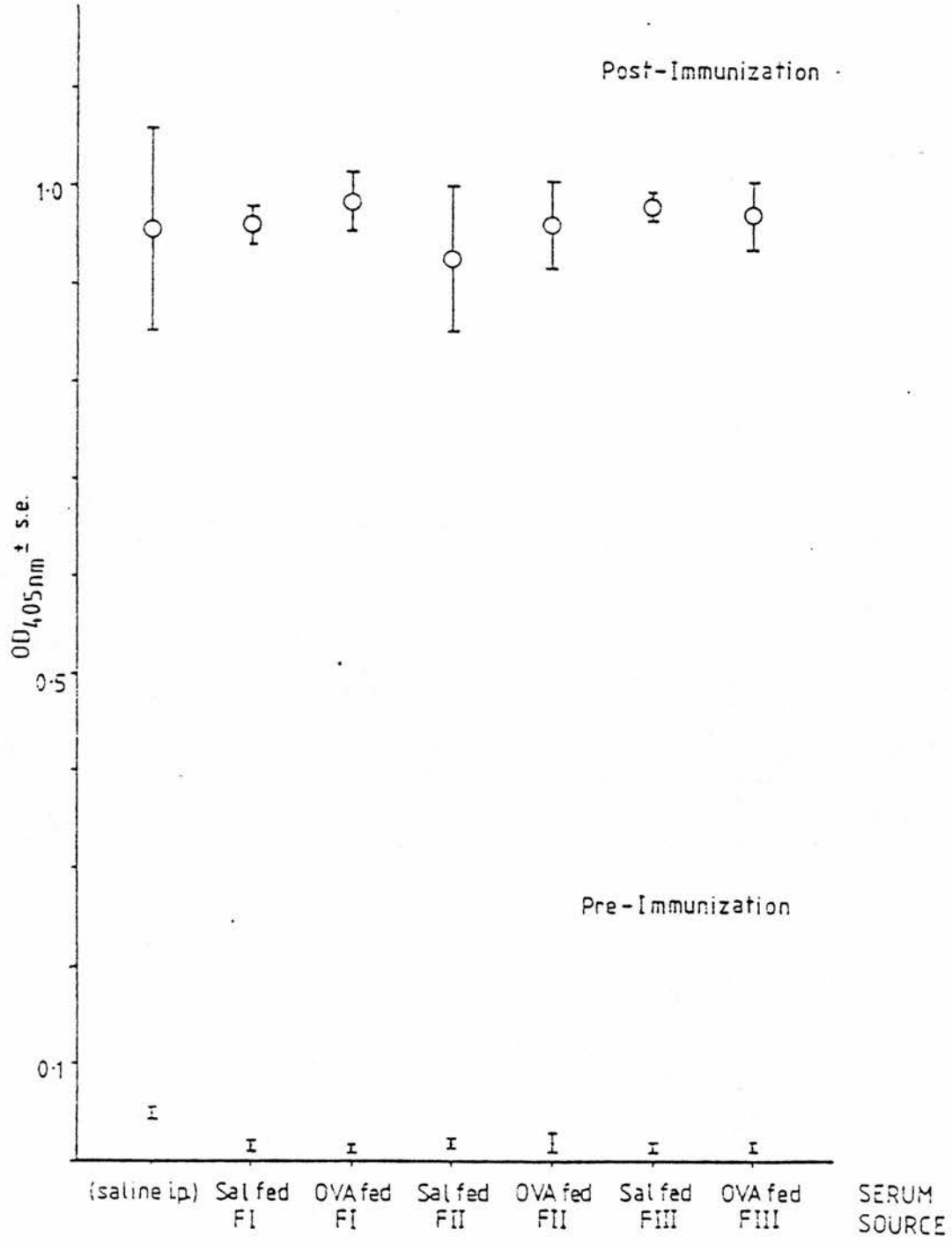

FIGURE 18

Pre and post-immunization antibody response in recipients of serum fractions from saline or OVA fed mice.

Serum was collected 1 hr after feeding and fractionated by gel filtration.

Recipients were injected with serum fractions from donors treated as shown and immunized with OVA 1 week later. The group indicated "(saline i.p.)" was injected with saline only and represents a normal positive response. Antibody (IgG) was measured by ELISA (OD 405 nm) 21 days after immunization.

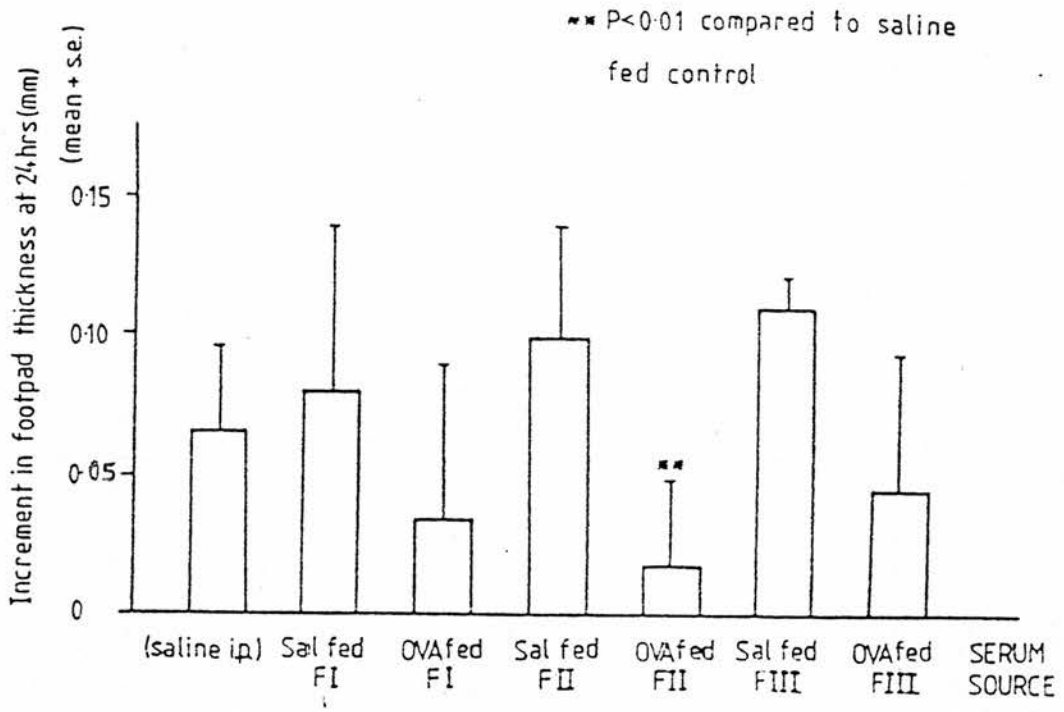

FIGURE 19

Cell-mediated response in recipients of serum fractions from saline or OVA fed mice.

Serum was collected 1 hr after feeding and fractionated by gel filtration.

Recipients were injected with serum fractions from donors treated as shown and immunized with OVA 1 week later. The group indicated "(saline i.p.)" was injected with saline only and represents a normal positive response.

DTH was measured by footpad swelling 24 hrs after antigen challenge. The response was assayed 21 days after immunization.

response was suppressed in recipients of serum fraction II from OVA-fed mice ($P < 0.01$ compared to "saline-fed" fraction II) Serum fractions I and III from OVA-fed mice did not induce significant suppression but produced lower DTH responses than those fractions from saline-fed mice.

7.5 Conclusions

The analysis of serum fractions revealed that no protein was detectable by Lowry technique in the low molecular weight fraction, FIII, of serum from saline-fed mice as expected. The identical result obtained with serum from OVA-fed mice implies that within the limitations of a Lowry assay, small fragments of OVA molecules are not present in serum at 1 hour after OVA feeding. Protein was detected in the high and "medium" molecular weight fractions of serum from both saline and OVA-fed mice. Ovalbumin however, was detected in FII of OVA-fed mice only.

At this stage, two possible hypotheses are presented: All OVA present in serum is in the fraction corresponding to native OVA and can be detected by antibody in the ELISA, or OVA which is not recognised by antibody is present in fraction I (high molecular weight).

On transfer into recipients, serum fractions did not suppress the humoral anti-OVA response of recipients. This is consistent with results obtained previously using whole serum.

Fraction II from OVA-fed mice suppressed DTH in recipients. Taken together with the analysis of fraction II by Lowry and ELISA techniques, this is consistent with the hypothesis that suppression of DTH by this fraction is due to the presence of OVA, bearing

determinants for anti-OVA antibody and having the same or approximately the same molecular weight as native OVA.

Fraction I and III from OVA-fed mice resulted in an observable lessening of the DTH response in recipients. Whilst not significant, this decrease in DTH does not entirely rule out the possibility of antigen being present in these fractions which, in the case of fraction III is not detected possibly by virtue of any of the following qualities; the molecule or fragment is too small or present in a concentration which is too low for its detection but which is still relevant in vivo or perhaps is lacking in the amino acids phenylalanine, tyrosine or tryptophan upon which detection in a Lowry spectrofluorometric assay depends. A lack of B cell determinants would explain the absence of immunoreactive OVA in both fractions I and III but this does not rule out the possible presence of immunologically active antigen in these fractions.

The effect of fraction II, suppression of DTH, appears to be the dominant effect which is seen when whole serum is transferred from OVA-fed mice.

CHAPTER 8

THE RELATIONSHIP BETWEEN INTESTINAL DAMAGE AND ANTIGEN PROCESSING

8.1 Introduction

Previous work described in this thesis (Chapters 4, 5, 6 and 7) investigated the nature and immunological properties of OVA which had been absorbed across the intestinal tract into the circulation. The purpose of the experiments described in this chapter was to establish the role of the intestine and its associated immune system in generating immuno-regulatory antigen in the serum by examining the effect of intestinal damage on the production of tolerogenic OVA in the serum after feeding.

Whole-body irradiation at a lethal dose of 1,000 rads was chosen as a method of damaging the gut. The effects of ionizing radiation on lymphocytes and on the intestinal epithelium are well-documented and were discussed in Chapter 1 of this thesis. The major effects of exposing the intestine to irradiation are lymphoid depletion with damage to the absorptive surface of villi followed by a period of regeneration when many new enterocytes are dividing in crypts and replacing the dead or damaged cells on villi.

The object of the following experiments was to study antigen processing in mice with intestinal damage in order to test the hypothesis that damage to the intestine alters the immunological properties of circulating gut-derived antigen. These in vivo experiments were carried out in parallel with analysis of body weight and intestinal histology and morphometry to determine the degree of radiation damage at 2 time-points following irradiation and also with ELISA tests on the serum of irradiated mice to assess absorptive function in the damaged intestine. Finally, the relevance of lymphoid cells to gut-processing was studied in irradiated mice which were reconstituted with spleen cells. This was an extensive study

involving large numbers of animals and was carried out in collaboration with Dr Stephan Strobel.

8.2 Experimental Design

Groups of BDFI mice were either left untreated or else given a total dose of 1,000 rads whole-body irradiation from an x-ray source. At 2 or 5 days after irradiation, the mice were weighed, given a single intragastric feed of either 25 mg OVA or 0.2 ml saline and were bled out 1 hour later. At this point, sections of jejunum were obtained and processed for histology and an aliquot of serum reserved for testing in an ELISA to detect OVA.

The remaining sera were pooled according to experimental groups and 0.8 ml serum injected i.p. into naive syngeneic recipients. These mice were immunized 1 week later with 100 μg OVA/FCA and were bled for serum antibody measurement and DTH-skintested three weeks later.

In experiments where mice were to be reconstituted with spleen cells, an injection of 25×10^6 naive syngeneic spleen cells in 0.05 ml RPMI 1640 medium was given i.v. within three hours of irradiation.

8.3 Effects of Irradiation in BDFI Mice

The mice lost weight following irradiation as shown in figure 20 and also exhibited changes in their gross physical appearance. Two days after irradiation the mice were quiet and subdued and by 5 days post-irradiation were quite lethargic and dehydrated, with diarrhoea. Less serum was obtained from these dehydrated animals.

At post mortem, the intestines of irradiated mice were very thin and shiny in appearance. Only tiny Peyer's patches were visible at 2 days after irradiation and none were seen by 5 days indicating that

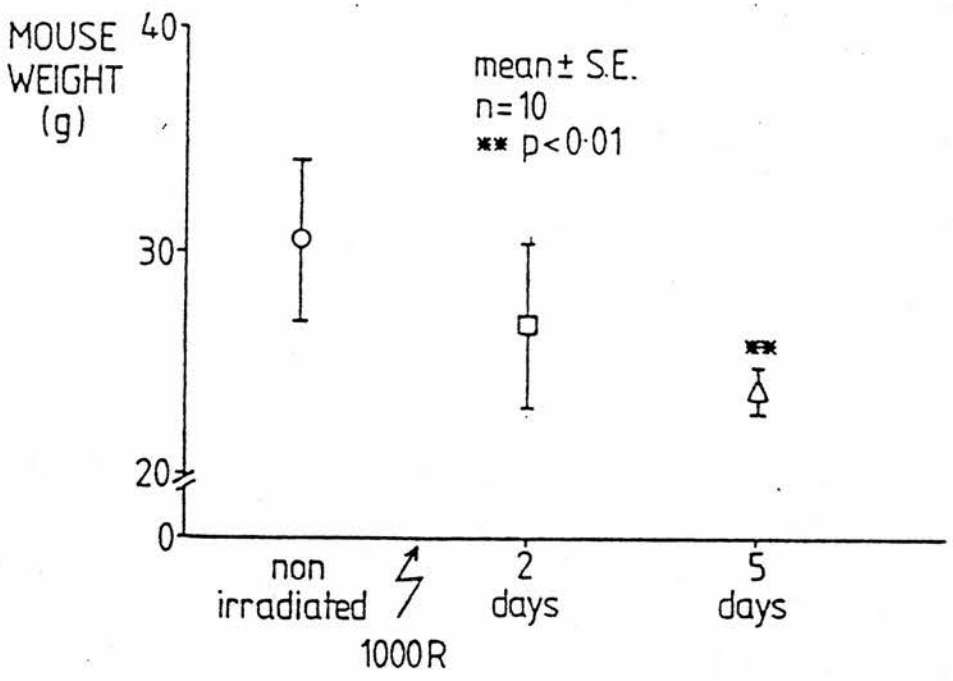

FIGURE 20

Effect of irradiation on weight of BDFI mice.

Mice were weighed before and at 2 and 5 days after 1000R whole-body irradiation.

severe lymphoid depletion had occurred in the gut-associated lymphoid tissue.

Histology of jejunal sections of gut, revealed that a significant reduction in villus length, together with crypt hypoplasia had occurred at 2 days after irradiation. The villus length was restored to normal by 5 days, and the crypts were longer than normal at this time indicating that the gut epithelium was in a state of regeneration. These points are illustrated in figure 21.

In addition to these morphometric changes in the appearance of the gut, the numbers of intraepithelial lymphocytes (IELs) decreased with time after irradiation, as shown in table 9. This agrees with the previous suggestion of lymphoid depletion in the GALT based on gross intestinal appearance.

8.4 Effects of Irradiation Damage on Intestinal Antigen Processing

Figure 22 shows the humoral antibody and DTH responses in recipients of serum from saline or OVA-fed mice which were either untreated or irradiated 2 or 5 days before feeding. This experiment is referred to as Series 1 in illustrations.

The antibody responses of all recipient groups were normal. On the other hand, while transfer of serum from non-irradiated OVA-fed mice suppressed the DTH response of recipients as is usual in an experiment of this nature, serum from both groups of irradiated, OVA-fed donors did not induce suppression of DTH in recipients and no significant difference was observed between these and recipients of serum from irradiated, saline-fed donors.

The OVA concentration in donor mouse serum was assayed in an ELISA. The serum of irradiated mice contained significantly more OVA

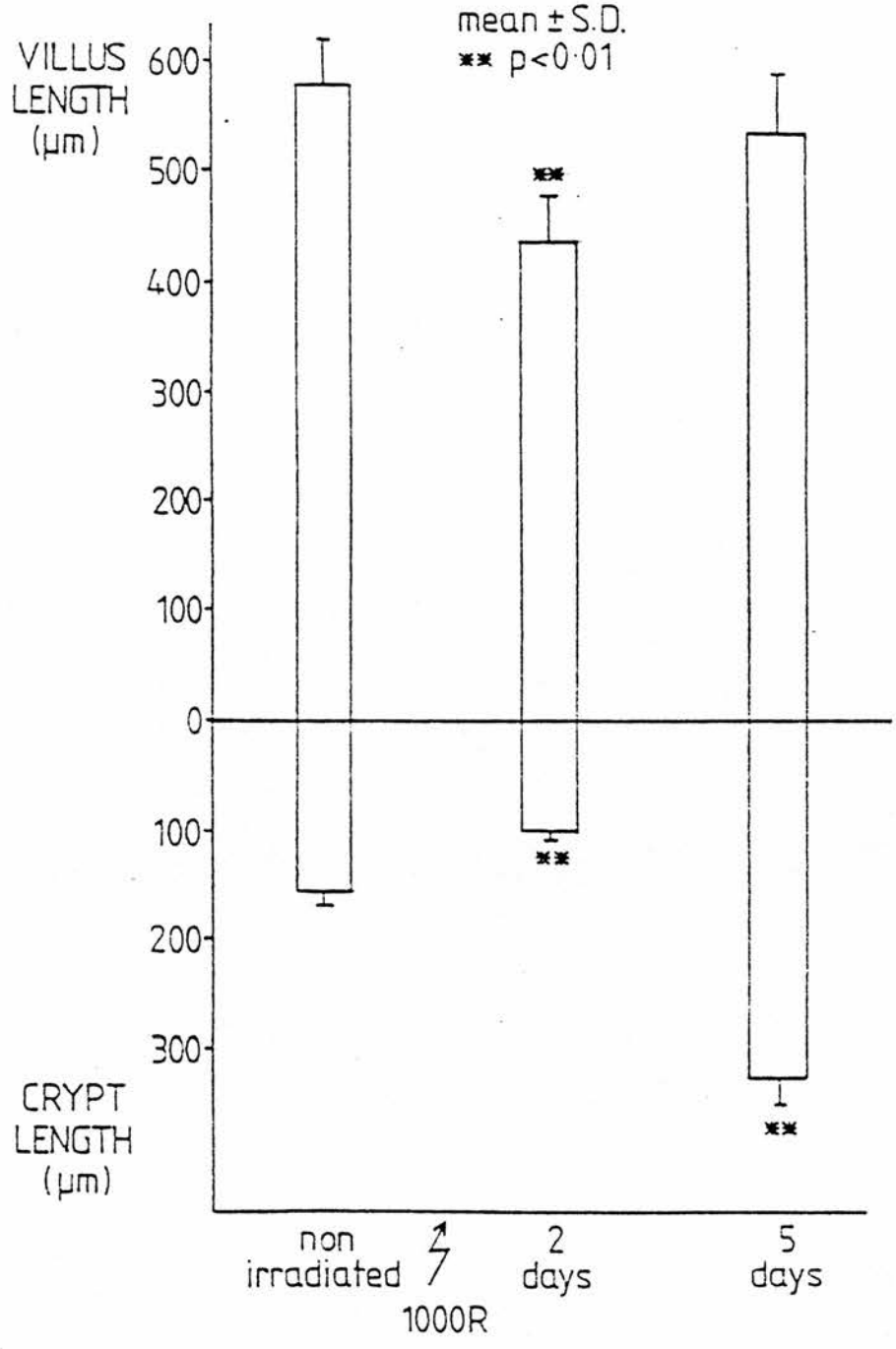

FIGURE 21

Effect of irradiation on jejunal villi and crypts of BDFI mice.

Measurements of villus and crypt length were taken from samples of jejunum collected before and at 2 and 5 days after 1000R whole-body irradiation.

	(IEL/100 epithelial cells ± s.d.)		
	Untreated	2 days post-irradiation	5 days post-irradiation
Series 1	11.3 ± 0.9 (n = 5)	7.0 ± 6.8 (n = 6)	4.4 ± 0.91 (n = 5)

TABLE 9

Effect of irradiation on numbers of jejunal intraepithelial lymphocytes in BDFI mice.

Mice were give 1000 R whole body irradiation.

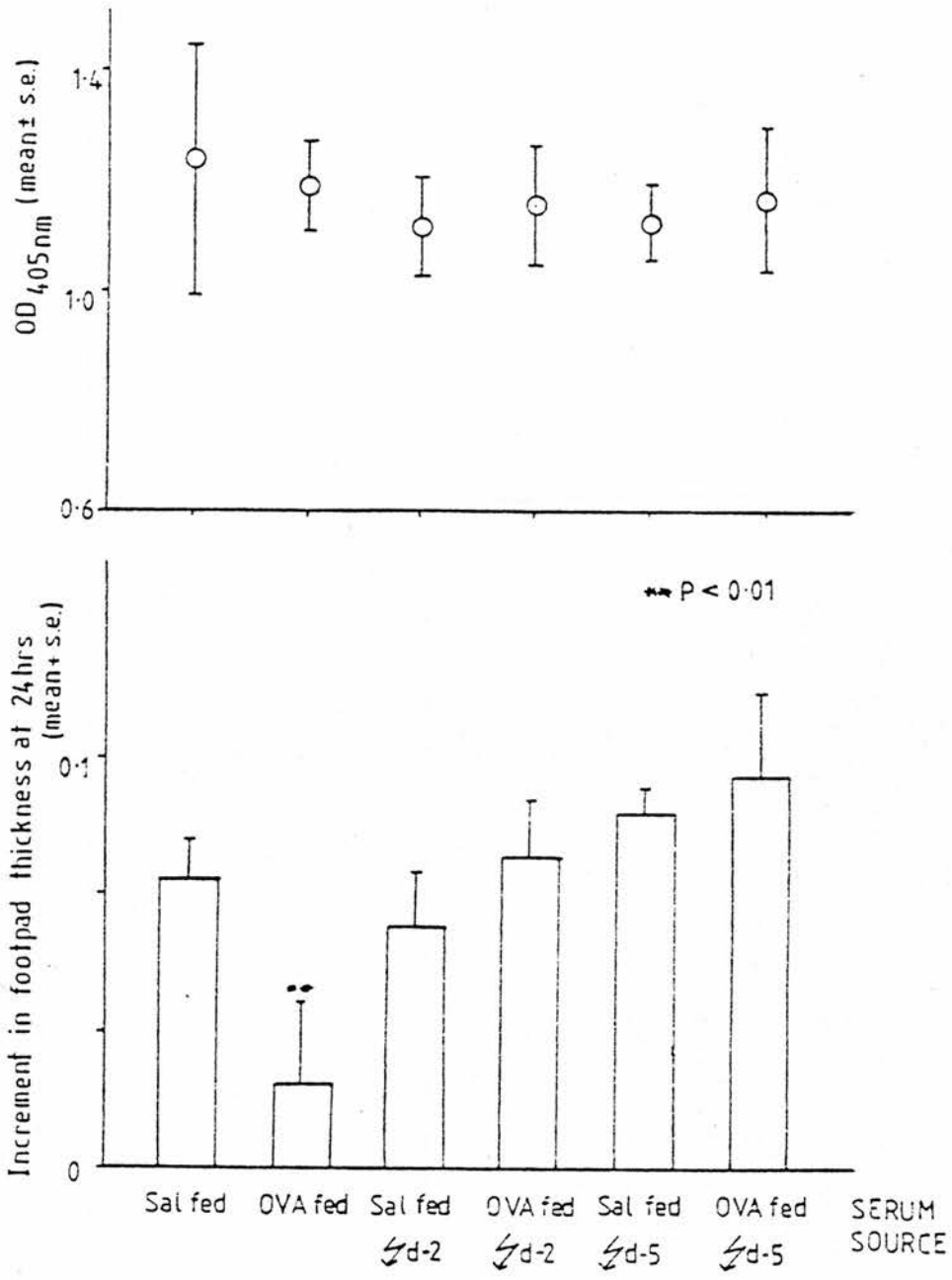

FIGURE 22

Systemic humoral and cell-mediated responses of recipients of serum from saline or OVA fed donors: Effects of irradiation of donors.

Antibody (IgG) was measured by ELISA (OD 405 nm).

DTH was measured by footpad swelling 24 hrs after antigen challenge.

Responses were assayed 21 days after OVA immunization.

SERIES 1: Recipients were injected with serum collected from donor mice one hour after sal or OVA feeding as indicated on day 0 and were immunized with OVA 1 week later. Donors were either previously untreated or had been irradiated 2 (ϟd-2) or 5 (ϟd-5) days before feeding.

		non-irradiated	2 days post irradiation	5 days post irradiation
OVA (ng/ml)	Series 1 mean ± s.e. n = 10	63.7 ± 50.2	228 ± 162 ($P < 0.01$)	235 ± 140 ($P < 0.005$)

TABLE 10

OVA (by ELISA) in Serum of BDFI Mice (each mouse fed 25 mg OVA one hour previously)

Mice were given 1000 rads whole-body irradiation.

than the non-irradiated controls (Table 10). Hence the absence of suppression of DTH in recipients of serum from irradiated mice was not due to any lack of ELISA-detectable OVA in the serum.

8.5 Effects of Irradiation and Spleen Cell Reconstitution on Intestinal Antigen Processing

Ovalbumin which was transferred in the serum of irradiated mice was not tolerogenic (Series 1). In order to examine the hypothesis that this had occurred as a result of the severe lymphoid depletion in irradiated mice, a serum transfer experiment was devised using, as serum donors, irradiated mice which were reconstituted with spleen cells on the day of irradiation (Series 2). Serum recipients were injected, immunized and challenged in the usual manner.

Figure 23 shows the mean body weight of donor mice before irradiation and at 2 and 5 days after irradiation and reconstitution. Alongside these values are the mean body weights of donor mice from Series 1. The pattern of weight loss following irradiation was identical regardless of whether reconstitution had taken place. It is worth noting that reconstituted mice also had improved general appearance with the only sign of dehydration seen at 5 days in some of the mice who presented with slightly scruffy coats.

The serum antibody and DTH responses of serum recipients in Series 2 are illustrated in figure 24. The results once again reveal no reduction in humoral antibody levels following serum transfer, and recipients of serum from non-irradiated OVA-fed mice again showed a suppressed DTH response ($P < 0.05$ compared to non-irradiated controls).

A novel finding however, was the suppression of DTH in recipients

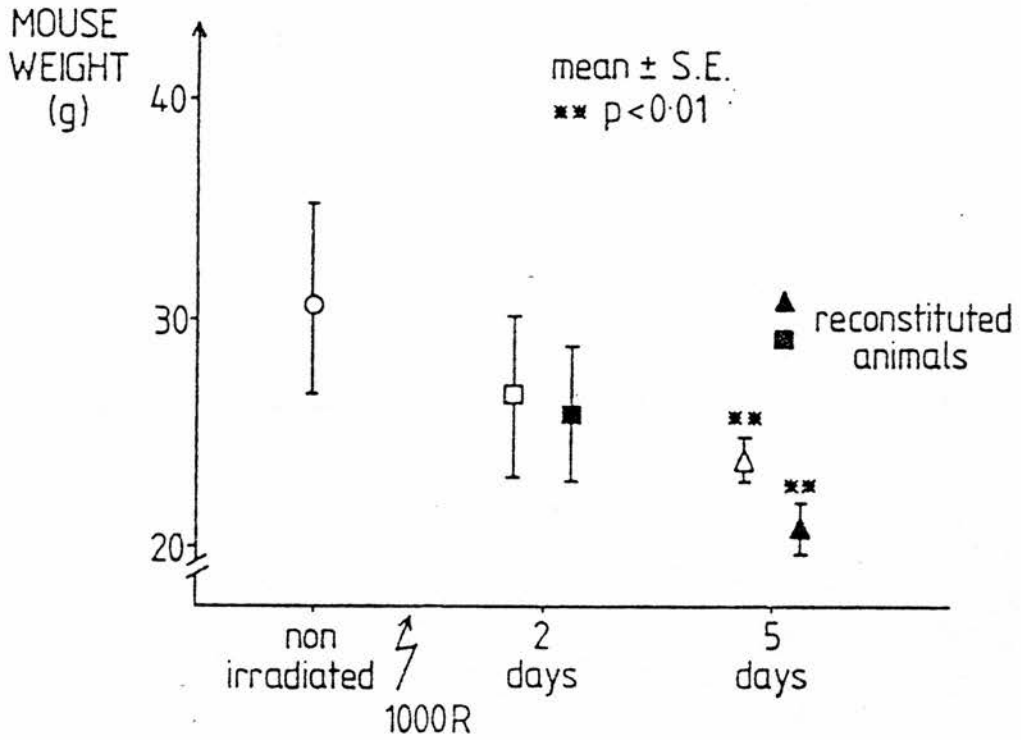

FIGURE 23

Effect of irradiation ± reconstitution on weight of BDFI mice.

Mice were weighed before and at 2 and 5 days after 1000R whole-body irradiation.

Shaded symbols represent mice which were irradiated and then reconstituted with 25 x 10^6 spleen cells within 3 hours of irradiation.

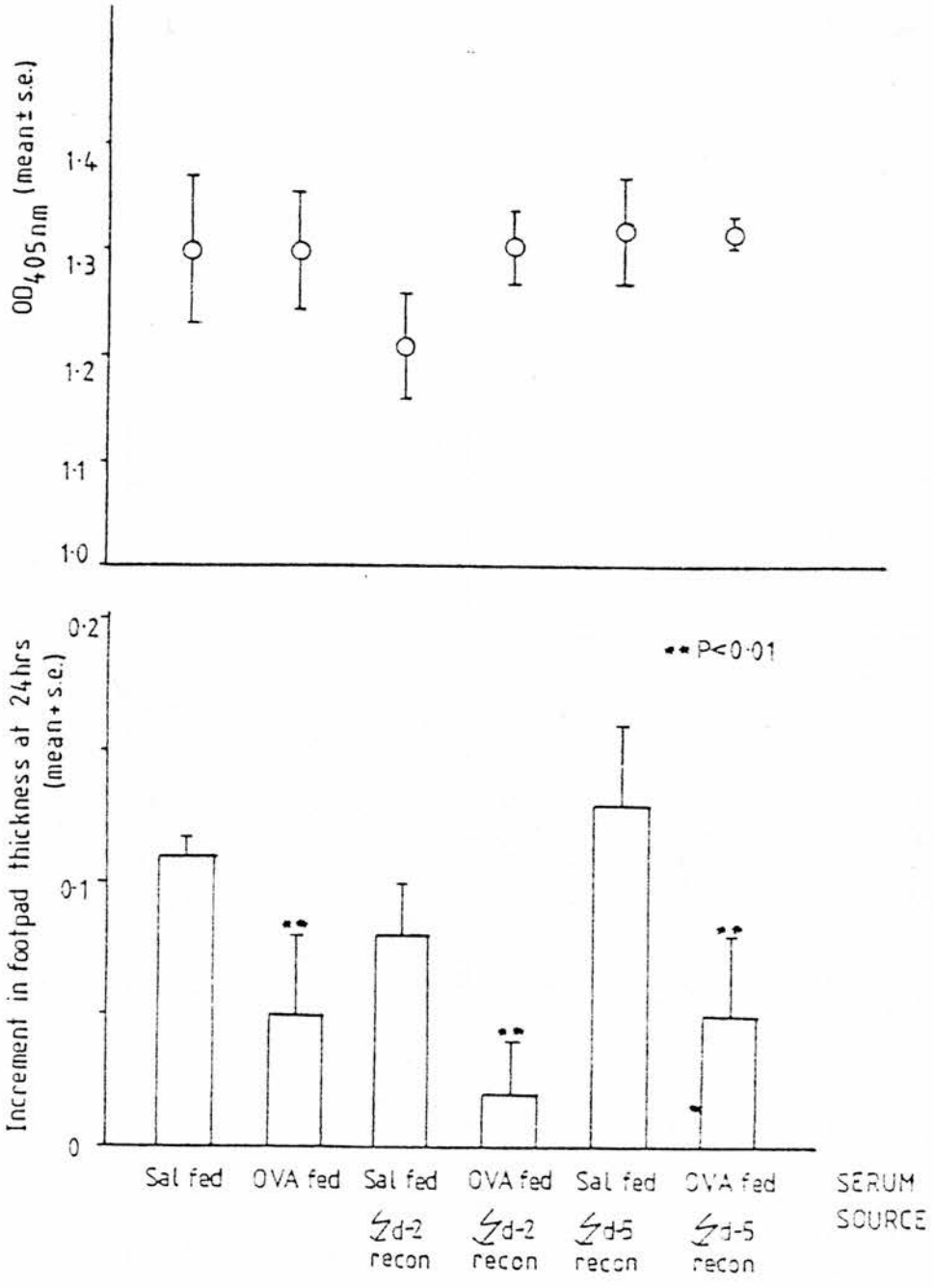

FIGURE 24

Systemic humoral and cell-mediated responses of recipients of serum from saline or OVA fed donors: Effects of irradiation and reconstitution of donors.

Antibody (IgG) was measured by ELISA (OD 405 nm).

DTH was measured by footpad swelling 24 hrs after antigen challenge.

Responses were assayed 21 days after OVA immunization.

SERIES 2: Recipients were injected with serum collected from donor mice one hour after sal or OVA feeding as indicated, on day 0 and were immunized with OVA 1 week later.

Donors were either previously untreated or had been irradiated and reconstituted with 25 x 10^6 spleen cells 2 ($\overset{\text{(recon.)}}{\text{ϟ}}$ d-2) or 5 ($\overset{\text{(recon.)}}{\text{ϟ}}$d-5) days before feeding.

of serum from OVA-fed mice which had been irradiated and reconstituted with spleen cells. Reconstitution with normal spleen cells following irradiation had restored the ability of donor mice to transfer tolerance for DTH with serum after OVA feeding.

This experiment was then repeated in combination with series 1, that is, using non-reconstituted mice, in order to obtain a more meaningful comparison of the effects of spleen cell reconstitution. Also in this combined test, which was called Series 3, serum obtained from donors was analysed by ELISA for immunoreactive OVA.

8.6 Comparison of the Effects of Irradiation of Serum Donors With and Without Spleen Cell Reconstitution

Groups of donor mice were irradiated and reconstituted with normal spleen cells in RPMI 1640 medium, or irradiated and sham-reconstituted with medium or else left untreated. Two days after irradiation, the mice were fed OVA and bled out after 1 hour at which point jejunal sections were collected and processed for histology. The pooled serum was tested for OVA and transferred into recipients which were later immunized and challenged with OVA as before. Note that only 1 post-irradiation time-point was selected. Since the effect of irradiation on serum-transferrable tolerance had been the same at both 2 and 5 days after irradiation, this practical step was taken to reduce the large number of animals required for the experiment.

The number of intraepithelial lymphocytes per 100 epithelial cells in jejunum was calculated for 2 donor mice per group and are shown in table II together with the IEL counts obtained in Series 1. The results indicate that the numbers of IELs decreased in jejunum following irradiation, as was the case in Series 1, and that the

numbers of these cells approached normal in irradiated mice which were reconstituted with spleen cells.

The humoral antibody and DTH responses of serum recipients in this combination experiment are summarised in figure 25. Serum antibody levels in recipients are not suppressed by serum transfer and are even elevated in three of the groups. The DTH response of recipients confirms the previous finding: Mice injected with serum from OVA-fed donors have suppressed DTH, although in this one experiment, the difference between this and the appropriate control group (recipients of serum from saline-fed donors) is not significant; irradiated donors do not transfer tolerance for DTH with serum after OVA-feeding but reconstituted irradiated donors do transfer tolerance with serum ($P < 0.05$ compared to recipients of serum from irradiated and reconstituted donors).

The OVA content of pooled sera from this test was measured in an ELISA and compared with the values obtained in the first experiment using only irradiated mice, (Series 1). The results of these assays are given in table 12 which reveals that the in vivo immunological effects of these sera (figures 22 and 25), occur over a wide range of OVA concentrations in serum and that overlap occurs in the amount of OVA present in "tolerizing" serum and "non-tolerizing" serum. For this reason it seems unlikely that the effects of irradiation with and without spleen cell reconstitution are the result of differences in the amount of OVA transferred in the serum.

8.7 Conclusions

Whole body irradiation removes the ability of mice to process OVA after feeding and render it tolerogenic. This is not due to a lack of

	(IEL/100 epithelial cells ± s.d.)		
	Untreated	2 days post-irradiation	5 days post-irradiation
Series 1	11.3 ± 0.9 (n = 5)	7.0 ± 6.8 (n = 6)	4.4 ± 0.91 (n = 5)
Series 3	12.1, 11.5	3.95, 5.0	—
Series 3 (mice reconstituted with spleen cells)	—	11.3, 11.1	—

TABLE 11

Effect of irradiation with and without reconstitution on numbers of jejunal intraepithelial lymphocytes in BDFI mice.

Mice were given 1000 rads whole-body irradiation.

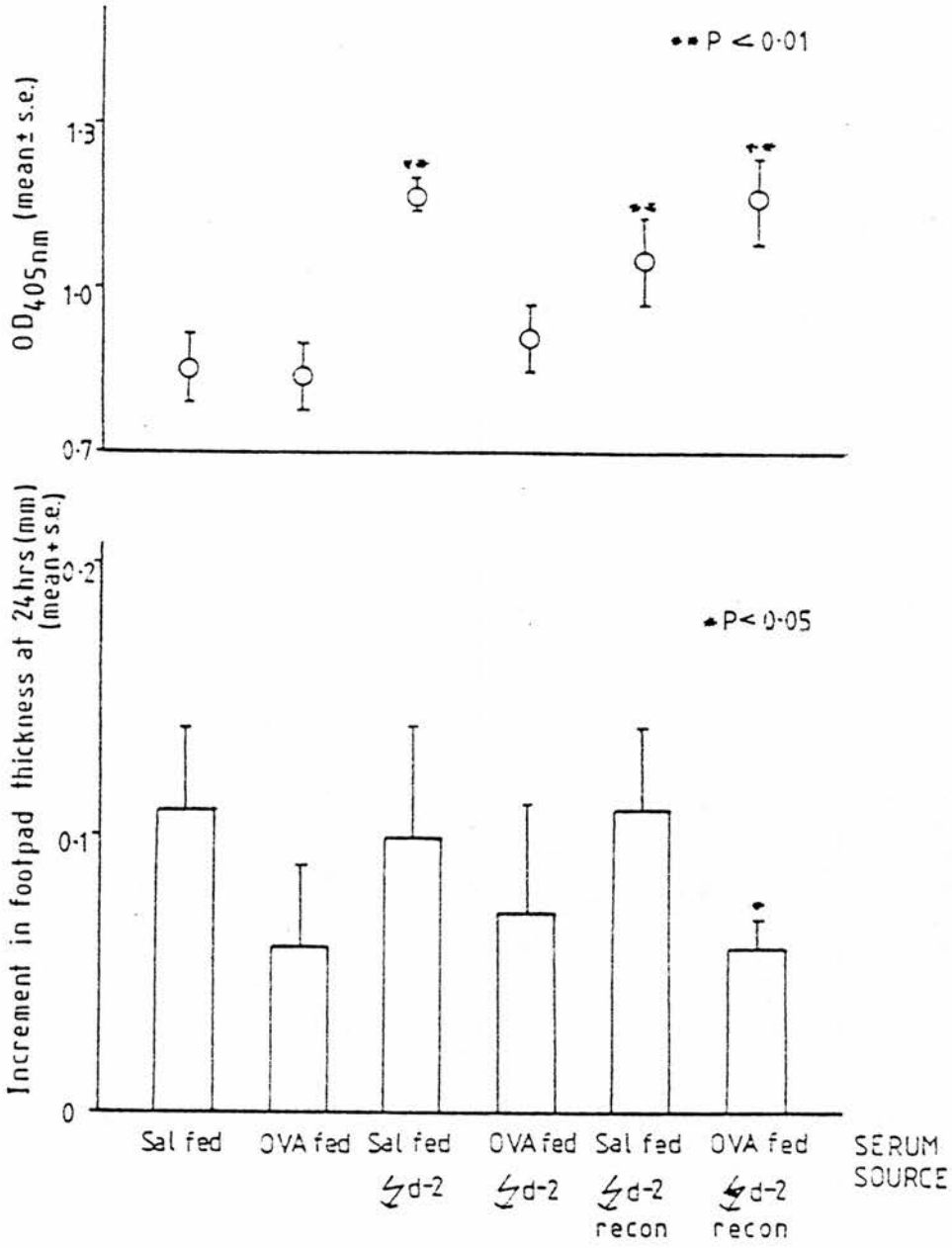

FIGURE 25

Systemic humoral and cell-mediated responses of recipients of serum from saline or OVA fed donors: Effects of irradiation ± reconstitution of donors.

Antibody (IgG) was measured by ELISA (OD 405 nm).
DTH was measured by footpad swelling 24 hrs after antigen challenge.
Responses were assayed 21 days after OVA immunization.

SERIES 3: Recipients were injected with serum collected from donor mice one hour after sal or OVA feeding as indicated, on day 0 and were immunized with OVA 1 week later.
Donors were either previously untreated, or irradiated 2 days before feeding (ϟ d-2), or irradiated and reconstituted with spleen cells 2 days before feeding (ϟ d-2 (recon.))

		non-irradiated	1000R ↯ 2 days	2 days (reconsti-tuted)
OVA (ng/ml)	Series mean ± s.e. n = 10	63.7 ± 50.2	228 ± 162	-
	Series 3 pooled sera n = 20	158.4	56.2	35.5

TABLE 12

OVA(by ELISA) in Serum of BDFI mice (each mouse fed 25 mg OVA one hour previously)

Mice were given 1000 rads whole-body irradiation as indicated.

antigen in the circulation of irradiated mice as an ELISA of OVA content in their serum revealed, in some cases, large amounts of immunoreactive OVA, nor is the lack of tolerance due to an alteration in the quantity of OVA present in the serum as the same in vivo effect is observed over a wide range of ELISA-detectable OVA concentrations in the serum of irradiated and non-irradiated mice (table 12). In addition, the effect of irradiation on serum-transferrable tolerance is found when the intestinal crypts are in either a state of hypoplasia (2 days post-irradiation) or hyperplasia (5 days post-irradiation) and so seems not related to a specific type of abnormality in the gut.

The irradiated mice were in a state of intestinal lymphoid depletion with sharply decreased IEL counts. After reconstitution with syngeneic spleen cells on the day of irradiation, the numbers of IELs in jejunum were restored to normal. Reconstitution with lymphoid cells also restored the ability of the intestine to process OVA such that the protein was once more tolerogenic in serum. As in the case of non-reconstituted irradiated mice, the presence or absence of tolerogenic OVA in the serum after feeding could not be correlated with the quantity of antigen in the circulation. I would suggest rather, that changes occur in the intrinsic physico-chemical properties of ovalbumin protein during intestinal processing and that this antigen processing is linked to the presence of a lymphoid compartment in the gut. In these serum transfer donors which are bled out after feeding, the lymphoid cells are not required to respond to OVA in order for tolerogen (for DTH) to be produced in the serum but are rather required as a facet of normal gut function in order for tolerogenic OVA to be present in the systemic circulation.

Further to this is the observation that irradiated and reconstituted mice continued to lose weight in the manner of non-reconstituted animals and suggests that restoration of gut antigen processing is not merely due to improvement in the general health and well-being of the irradiated mice after spleen cell reconstitution but is due to the presence of healthy lymphoid cells. Such an association between the gut and GALT would be a natural link between normal intestinal function and immune regulation induced by feeding.

CHAPTER 9

GENERAL DISCUSSION

9.1 Introduction

The induction of specific systemic immunological tolerance in mice by feeding a single dose of the soluble protein ovalbumin has been the subject of study in this thesis. The phenomenon of oral tolerance is a phenotypic response, that is, the net host response occurring as the result of inter-reactions between the intestinal tract, the gut-associated lymphoid tissue and the systemic immune system.

In the search for an underlying mechanism for this phenotypic response, many workers have studied only a single aspect of the response and often by indirect methods such as plaque-forming cell (PFC) assays. Counts of the numbers of plaque-forming cells in spleen and GALT bearing surface antibody of particular isotypes have provided information about the regulation of B cells following antigen feeding.

Mattingly and Waksman transferred into recipient animals, cells from Peyer's patches, mesenteric lymph nodes, spleen and thymus of rats at various times after feeding sheep erythrocytes. They described suppressor cells for IgM and IgG PFC responses which were generated in the GALT of donor rats after 2 days of antigen feeding and then migrated to the spleen and thymus where they were found at 4 days after antigen feeding (Mattingly and Waksman 1978).

Similar work in mice showed that the continuous feeding of heterologous erythrocytes initially primed the IgA and IgG PFC response in the spleen but suppressed the splenic IgM PFC response (Kagnoff, 1978 b). In addition, suppression of IgG and IgA PFC responses by Peyer's patch cells was found to be due to the presence of suppressor T cells which were effectively masking the concomitant

priming of B cells which could respond when the suppressor population was removed (MacDonald, 1983).

Evidence of isotype-specific regulation of B cells by feeding a protein antigen came from Richman et al, (1981) who demonstrated the presence of both IgA-specific helper T cells and IgG-specific suppressor T cells present in Peyer's patches within 1 day of feeding. These cells subsequently migrated to the mesenteric lymph nodes where they were detected at 3 days after feeding. After this time the IgG-specific suppressor cells were found in the spleen but the helper cells for IgA did not apparently leave the GALT.

Whilst these experiments have highlighted particular regulatory mechanisms induced by antigen feeding it is not clear how well the PFC response reflects the net humoral antibody response. On this point it is interesting to note that circulating antibody can be detected in the absence of plaque-forming cells (Thomas and Parrot, 1974).

Many other workers have measured circulating specific antibody following antigen feeding and the suppression of the humoral response following the ingestion of antigen is a well documented phenomenon. However experiments described in this thesis have shown that tolerance for cell-mediated immunity can occur in the presence of a normal serum antibody response (Chapter 4) and emphasises the importance of experiments designed to assay more than just one component of the systemic response.

In the course of designing such experiments, difficulties in assessing the regulation of cell-mediated immunity arise. The use of in vitro techniques such as measuring the proliferation of cells in response to a specific antigen have been useful in defining cells capable of mediating the regulation of systemic immunity by feeding.

The experiments of Miller and Hanson (1979) on BDFI mice fed with OVA demonstrated that suppression of systemic DTH, measured *in vivo* as an ear swelling response, correlated well with the *in vitro* proliferative response of cells from peripheral lymph nodes. In my experiments (Chapter 3) I was unable to correlate the *in vitro* proliferative response of BDFI mice which were fed OVA with the *in vivo* expression of DTH in a footpad skintest. That is, despite the presence of a suppressed DTH response *in vivo*, I was unable to discern a concomitant significant decrease in the *in vitro* response. Whether this result was due to differences in technique as discussed in chapter 3, or whether different populations of cells are involved in ear swelling and footpad thickening in response to local antigen injection is unknown at present.

Selective depletion of cells in culture has been used as a tool to identify subsets of regulatory cells operating *in vitro*. For example, by removing T cell sub-populations from peripheral lymph node cells, Silverman *et al* (1983) were able to show that suppression of the proliferative response of lymph node cells from mice fed with BSA prior to immunization was mediated by a T cell bearing Ly 1^+ 2^- surface antigens.

Mucosal immunization with a viral antigen also induces production of Ly 1^+ 2^- suppressor T cells which suppress DTH when transferred *in vivo* (Liew and Russell, 1980) and although Ly 1^+ 2^- is not a surface marker traditionally associated with suppressor cells, which were thought to be of Ly 2^+ phenotype (Feldmann and Kontiainen, 1976), this may represent a particular regulatory cell induced by feeding.

Results from *in vitro* experiments must be applied *in vivo* with caution, since it is by no means established that a particular

cellular mechanism which operates *in vitro*, perhaps only as described above, following the removal or the addition of other regulatory cells, is the responsible mechanism for an *in vivo* phenotypic response or even that such a mechanism operates at all *in vivo* since observations of a state of tolerance do not always correlate with studies of individual cellular function (Parks and Weigle, 1980 a ; Hanson and Miller, 1982).

Therefore, while *in vitro* studies of the sort discussed above are useful in defining isolated mechanisms of immune regulation, experiments to assess the *in vivo* responsiveness of whole animals (although they are often time-consuming to perform and complex to design and interpret) are still necessary if a logical arrangement of the various regulatory mechanisms described so far into a net phenotypic response is to be achieved.

Using the serum transfer protocol, I examined the role of the gut in the induction of tolerance following antigen feeding by collecting the antigen itself immediately after its passage across the gut mucosa, and studying its behaviour both *in vivo* and immunochemically. In these types of experiments it was possible to dissect out the systemic component of the immune response to antigen entering the circulation via the gut both in normal animals and in immunologically-compromised hosts treated with cyclophosphamide.

In these experiments I adopted a protocol of measuring systemic DTH and humoral antibody. With hindsight, it would also have been of value to examine the isotype of antibody produced by serum recipients since an overall positive presence of serum antibodies might mask the suppression of a particular immunoglobulin isotype.

The ELISA for detecting OVA-specific antibody measured only IgG

anti-OVA. The indirect haemagglutination assay which I used at the start of my project was time-consuming and required much larger sample volumes than the ELISA but did offer the option of estimating the relative amounts of IgG and mercaptoethanol-sensitive antibody (presumed IgM). In order to detect antibody isotypes by ELISA it would be necessary to develop a separate assay for each isotype using specific anti-isotype antibodies for their detection. These assay methods have recently been achieved in this laboratory and therefore analysis of stored serum samples will be carried out in the coming months.

The footpad skintest, which was my choice of method for measuring systemic DTH, is a fairly crude, if simple, technique and should ideally be used in conjunction with some other test of cell-mediated immunity such as passive transfer of DTH effector cells plus antigen into the footpad of a naive recipient mouse: The recipient acts merely as a living incubator and cellular proliferation in response to antigen is seen as an increase in footpad thickness (Kettman and Mathews, 1976).

Another test of cell-mediated immunity is the in vitro cell proliferation test. I attempted to use this assay in conjunction with the DTH skintest but met with only limited success as I have already described.

Using the footpad skintest for DTH, reproducible results can be obtained with practise and partly for this reason it was important for me to test that I could repeat the earlier established experiments of Mowat (Mowat et al, 1982) in which mice were parenterally immunized (positive for DTH), or were tolerized by feeding OVA (suppressed DTH), or had oral tolerance abrogated by pretreatment with CY (positive for

DTH). I found that I was able to reproduce these results in BALB/c mice (Chapter 3) and was therefore confident in my interpretation of subsequent experiments involving skintesting for the estimation of systemic DTH.

The importance of the intestine and its associated immune system in the regulation of immunity by feeding was explored by studying the intestinal handling of a protein antigen under normal and abnormal conditions such as after CY treatment and following irradiation.

The antigen which I used was five times recrystallised ovalbumin from chicken egg white (OVA) and there were inherent advantages in using this protein. Since many other workers have studied the same antigen, there exists a large body of published data for comparison with my own work. In addition, although egg protein such as OVA was not a typical dietary antigen for my experimental mice, it is of interest that for many humans, egg albumin is a common dietary protein and has been identified in egg white as a major allergen for humans (Langeland, 1982). OVA is therefore of interest to workers studying both human disease and animal models of immunity.

Many antigens commonly introduced into the intestine are not protein, such as bacterial antigens for example, and so it would be unwise to form a unifying hypothesis about the regulation of responses by feeding "antigen" without considering that various types of antigen are commonly encountered by the gut and may produce distinct patterns of immunity.

Even within the body of data relating to different protein antigens used by other workers, results should not be compared without realising that differences in protein structure may result in differences in digestibility for instance, and in antigen processing

by the intestine which can in turn influence the regulation of the immune response to a particular protein. In the context of this argument OVA may not be the most suitable protein to use in feeding experiments as it has some distinctive properties such as being heavily phosphorylated and bearing a large carbohydrate moiety which make it rather unusual among proteins (Vadehra and Nath 1973). However, in immunological experiments such as mine, it was vital to introduce an antigen which had never been previously encountered by the experimental mice and which was simple to dissove and feed in measured doses and so for these reasons I made the decision to use this readily available antigen.

A disadvantage in buying a commercially prepared antigen is the risk of impurity. However, rabbit anti-OVA antiserum raised against five times crystallised OVA formed a single precipitin line in agarose gel when tested with OVA. Therefore, if five times crystallised OVA is contaminated to a slight degree with other egg white proteins, I was content that OVA was the immunodominant antigen and that the effects I was observing were due to the immune response to OVA and were not influenced to any noticeable extent by contaminants. I certainly felt that in view of the time allotted for the project, the size and number of experiments and the amount of antigen required, that it would have been impractical to continually assess the purity of my antigen or to attempt to purify OVA in the laboratory. I compromised by always ordering from the same company and always ordering the same type of antigen preparation.

9.2 Suppression of Cell-Mediated Immunity by Serum Transfer

In previous serum transfer experiments by other workers investigating the suppression of antibody responses, various antigens have been used including OVA and a particulate antigen, sheep erythrocytes. Serum was collected at several different times after antigen feeding; from 8 hours to 14 days, and a consistently negative result was achieved in all cases. Serum transfer after antigen feeding did not suppress antibody responses in recipients (Hanson et al, 1979; Kagnoff, 1978 b). The only report of the transfer of tolerance by serum from mice given a single feed of OVA comes from my own laboratory (Strobel et al, 1983).

After discussion with Dr Donald Hanson and based on his experience with serum transfer experiments, serum was collected one hour after feeding 25 mg OVA. This was a suitable period to allow for gastric emptying and protein absorption to occur (D Hanson, personal communication, 1980) and was a convenient interval in which to feed several mice before it became necessary to bleed out the first mouse. Serum obtained in this manner was capable of inducing specific suppression of the cell-mediated arm of immunity.

The one hour post-feeding serum collection was used for all the serum transfer experiments. This was done for practical reasons; a serum transfer experiment, taking serum at a single time point involved feeding and bleeding very large numbers of mice in order to obtain enough serum and this part of the experiment took several hours. It would have been unrealistic to attempt to collect sera at different time points with the resources available including time and technical assistance. However, in view of the data generated by these experiments it would now be of great interest to examine the

immunological effects of sera obtained at other time points after feeding.

The work of Swarbrick on the uptake of radiolabelled OVA after a single feed suggested that absorption was biphasic with peaks in OVA concentration at 45 minutes and at 70 minutes (Swarbrick, 1979). To investigate the immunogenicity of absorbed antigen even at just these two times would be of interest, as would a much later collection of serum, perhaps not as late as 24 hours after feeding since by this time there is evidence of a host immune response to the ingested protein both from my own work (Chapter 3) and that of others (Silverman et al, 1983), but certainly after several hours. The relative amounts of postulated immunogenic and tolerogenic antigen present in serum after feeding may well be quite different at different times following antigen feeding and it would be interesting to test the net immunological effect of any remaining antigen or fragments of antigen.

The humoral antibody response was not suppressd by serum transfer. This agrees with the published results just mentioned and illustrates the importance of an experimental design which assesses both the humoral and cell-mediated arms of an immune response.

In experiments where tolerance has been transferred by serum from mice fed with several doses of a particulate antigen, the serum has been found to contain either antibodies (Kagnoff 1978 c) which may even be anti-idiotypic in specificity (Kagnoff 1980), or immune complexes (André et al, 1975). All of these are the result of host immune responses directed against the fed antigen. In my experiments, where serum was taken only one hour after feeding, it is unlikely that any such products of an immune response could accumulate in this short

space of time.

Further evidence for this has come from experiments performed by other workers in this laboratory: Mice were fed 25 mg OVA and were bled out after one hour. Their serum was injected into naive syngeneic recipients which were in turn bled 7 days later. Their serum was then tested for the presence of anti-OVA antibodies. No such antibodies were detected, and so mice injected with serum containing gut-processed OVA did not mount an anti-OVA antibody response, and more importantly, nor did they possess any antibodies passively transferred with the serum of OVA-fed mice (Strobel et al, 1983). For this reason it seems unlikely that my observation of tolerance transferred by serum from OVA-fed mice is due to the transfer of products of a host immune response such as antibody or immune complexes.

A somewhat unusual observation was the induction of tolerance for CMI with serum from OVA-fed mice whilst humoral immunity in recipients was apparently unaffected. This strengthens the theory that multiple mechanisms are involved in immune regulation following antigen feeding. In addition, serum-transferable tolerance for CMI is abrogated if the recipients are injected with 100 mg/kg cyclophosphamide 2 days prior to injection with serum. My initial experiments with BDFI mice showed that the reversal of oral tolerance by CY was restricted to the suppression of CMI (Chapter 3). Previous work with CY, described in Chapter 1, has borne out the particular effect of this drug on the regulation of cell-mediated immunity by suppressor cells. Briefly, CY will reverse tolerance which is caused by suppressor cells (Turk and Parker, 1982; Sy et al, 1977) and when used within the dose range of 20 - 200 mg/kg, has been

found to inhibit short-lived suppressor cells affecting DTH responses (Diamantstein et al, 1981; Atallah et al, 1979).

My experiments, demonstrating the induction of tolerance in BDFI mice by feeding 25 mg OVA and the restoration of DTH by CY pretreatment have not shown, other than by inference, that suppressor cells are responsible for the induction of oral tolerance and future work involving cell transfers should clarify this. Miller and Hanson (1979) were able to demonstrate by means of sequentially timed spleen cell transfers from BDFI mice fed 20 mg OVA, that tolerance for DTH was induced by suppressor cells acting on the afferent limb of the response. In addition, work from this laboratory has shown that treatment of serum donors with CY does not affect either the uptake of antigen or the appearance of DTH tolerogen in the serum (Strobel et al, 1983) and the morphological and histological studies of Mowat (1981) showed that CY did not appear to alter epithelial cell structure. This is strong evidence in favour of the hypothesis that my observations of CY-sensitive tolerance for DTH, induced by an injection of 0.8 ml serum collected one hour after OVA ingestion, is due to the activation of a distinct class of suppressor cells which do not act on antibody responses.

Whisler and Stobo have previously described a mouse suppressor cell for DTH which did not inhibit plaque-forming cell responses (Whisler and Stobo, 1978). In serum recipients which in my experiments are injected with serum parenterally, such a suppressor cell could presumably be activated at a site distant from the GALT. Whether this is true of intact animals tolerized directly by feeding antigen is uncertain.

BDFI mice fed with 25 mg OVA develop tolerance for both DTH and

humoral antibody responses. Collecting the antigen in serum one hour after such an oral dose of OVA and transferring this into naive mice effectively bisects the compound regulation induced by feeding. The serum recipients are exposed to a tolerogenic stimulus for DTH but not for antibody responses. This implies that antigen recognition in the gut-associated immune system is necessary for the combined suppression of both cell-mediated and humoral immunity.

I have already postulated a role for suppressor cells in the induction of oral tolerance for CMI. When considering such immunoregulatory cells in the immune system of the gut, there have been several descriptions of GALT suppressor cells by other workers. Ngan and Kind (1978) reported the presence of T cells in the Peyer's patches and spleens of mice fed OVA which suppressed specific anti-OVA IgE and IgG responses when transferred into recipients. They also noted that Peyer's patch suppressor cells produced a more profound, long lasting suppression than splenic suppressor cells. Cell transfer experiments in rats fed with sheep erythrocytes also established the presence, in Peyer's patches, of suppressor cells for IgM and IgG responses to a particulate antigen and demonstrated GALT suppressor cells for DTH responses (Mattingly and Waksman, 1978).

Later work by Challacombe and Tomasi (1980) demonstrated suppression of CMI (measured as a proliferative response of cells in vitro) in cells taken from GALT after feeding either protein or a bacterial antigen. While the recent experiments of MacDonald showed that in mice fed with sheep erythrocytes, B cells in Peyer's patches were primed by the antigen but were unable to respond due to the presence of suppressor T cells which prevented the generation of IgG and IgA plaque-forming cells (MacDonald, 1983).

If such a regulatory mechanism exists in the Peyer's patches of mice fed with OVA, and there is some evidence for suppressor cells controlling at least IgG anti-OVA formation in mouse Peyer's patches (Richman et al, 1981), it could be postulated that gut-processd antigen would tend to prime B cells and that these cells would be inhibited by GALT suppressor cells. In serum transfer experiments, where gut-processed antigen is injected i.p. such that Peyer's patch suppressor cells for B cell responsiveness are not generated in recipients, one might predict the already observed outcome of the induction of a systemic humoral antibody response with the concomitant induction of systemic suppressor cells for DTH responses. However, I observed no evidence of priming for antibody responses by serum transfer and the hypothesis would require to be tested by means of experiments involving serum transfers followed by cell transfers in combination.

Antigen dose is an important factor governing immune regulation and so it is appropriate to consider the effective dose of OVA presented to serum recipients in my experiments. An oral dose of 2 mg OVA suppressed CMI but not antibody responses in BALB/c mice (Mowat et al, 1982) and the difference in immunological outcome of oral versus parenteral exposure to oxazolone had been attributed to a difference in the amount of systemically available antigen in oxazolone-fed animals which led to the induction of tolerance (Asherson et al, 1979). Likewise, the work of Hanson on the induction of antibody tolerance to soluble proteins in newborn mice produced good evidence that regulation of the antibody response was determined by the dose of fed antigen; a small dose of 0.1 µg of HGG per gram body weight resulted in priming while 1 µg/g produced no net effect and larger

doses of 10 - 1,000 μg/g induced tolerance (Hanson, 1981).

My experiments have shown that parenteral administration of native OVA within a range of doses typically found in the circulation after feeding (0.01 μg - 10 μg), does not induce tolerance or priming for either DTH or antibody responses (Chapter 5), and measurements of the amount of OVA present in tolerizing and non-tolerizing sera (Chapter 8) revealed that there is no apparent relationship between the amount of OVA transferred in serum and suppression of DTH.

However, the effect of large doses of gut-processed antigen on antibody responses has not been tested, and while my results suggest that the quantity of antigen transferred in serum after feeding is not relevant to the induction of tolerance for DTH in recipients, it would be interesting to examine whether a substantial increase (perhaps 1,000 μg) in the amount of gut-processed OVA transferred would induce tolerance for antibody also.

The work of Miller et al (1979) on the induction of tolerance with protein antigen coupled to syngeneic lymphoid cells had shown that the route of parenteral antigen injection could influence the immune response. Subcutaneous injection resulted in the antigen reaching the circulation via lymphatic drainage, presumably being exposed to cells of the immune system in lymph nodes, and tended to induce immunological priming. On the other hand, antigen which was injected intravenously preferentially induced tolerance. In my experiments with a soluble protein antigen, I found no difference in the response to a range of doses of native OVA whether administered directly into the circulation by an i.v. injection or via the intra-peritoneal route by which antigen may be cleared by a combination of direct venous drainage or via lymphatic channels

(Chapter 5).

Similar doses of gut-processed OVA were injected i.p. in serum transfer experiments and induced tolerance. It seems likely therefore, that the induction of tolerance with serum containing gut-processed soluble antigen is not dependent on whether the route of injection is intravenous or intraperitoneal.

Other workers studying regulatory mechanisms induced in oral tolerance have used various strains of mice for their experiments. In the experiments described in Chapter 3 of this thesis, I repeated the oral tolerance experiments of Mowat with BALB/c mice (Mowat et al, 1981) and used an identical protocol in BDFI mice to test the induction of oral tolerance by a single feed of 25 mg OVA and the sensitivity of this phenomenon to CY. Unlike BALB/c mice, CY-sensitive suppression is restricted to DTH responses in the BDFI strain. Oral tolerance for serum antibody production in these mice is therefore resistant to CY and thus seems likely to be under the control of a different regulatory mechanism in mice of this strain. Strain-related differences in responsiveness to orally administered antigen such as I observed between BALB/c mice and BDFI mice are not well understood and highlight one of several areas of mucosal immunology requiring further study.

In one recent report however, Stokes et al compared antigen absorption and oral tolerance induction in 3 genetically distinct strains of mice. They found that not all strains exhibited immune exclusion to fed antigens after a previous oral immunization and that immune exclusion and oral tolerance were not associated phenomena. In addition, although all 3 strains tested were able to develop oral tolerance to fed antigen, 2 of the strains were only made tolerant if

the antigen was fed continuously in the drinking water as opposed to via a single intragastric intubation (Stokes et al, 1983).

A genetic basis for inter-strain differences in gut-related immunity should not indicate a requirement for immunologists to design experiments testing purely Ir gene function. Clearly, a difference in intestinal function due to some other set of genes unrelated to the immune system, perhaps influencing antigen handling in the gut, could well affect the ultimate immune response to an ingested antigen.

In addition, strain differences in intestinal immunity could well reflect differences in gut flora or susceptibility to enteric infections such as viruses or protozoa for example. These situations also might subsequently affect the net host response to antigen in the intestine.

The sex or age of an animal may be related to oral tolerance and although studies of immune regulation by feeding exist which describe newborn animals, information is scarce regarding the influence of ageing or gender on immune regulation by antigen feeding and further study in this field also is obviously required.

9.3 Nature of the Circulating Tolerogen Produced by Ovalbumin Feeding

The induction of tolerance by an i.p. injection of gut-processed OVA in serum is not related to the effective systemic antigen dose, nor is it associated with a particular route of injection. It is probable that the nature or quality of gut-derived OVA is relevant to its tolerogenic properties. The serum tolerogen could be produced by physico-chemical alterations to the native protein in the intestine occurring as part of the normal assimilation of exogenous proteins. Examples of published data described in Chapter 1 have shown that structural modification can dramatically alter immune responses to the same antigens.

Systemic exposure to denatured and to deaggregated OVA over a range of doses comparable with the concentration range of gut-processed OVA in serum, revealed that processing of OVA after feeding was not equivalant to protein denaturation since, unlike gut-processed OVA, this form of antigen had no effect on the systemic immune response to native OVA (Chapter 5). Previous work had shown that denatured protein antigens were capable of modifying the immune response directed against the native protein (Goetzl and Peters, 1972; Takatsu and Ishizaka, 1975).

Deaggregated OVA on the other hand was a potent tolerogen and this agrees with previous published data (Dresser, 1962; Golub and Weigle, 1969). However, deaggregated OVA induced suppression of both DTH and antibody whereas the gut-processed antigen normally only induced tolerance for DTH. It seems unlikely therefore, that gut-processed OVA is simply deaggregated OVA. Other workers have shown that "impure" preparations of deaggregated proteins, that is when the protein was not wholly deaggregated, were inefficient at

inducing tolerance for antibody production as macrophages were able to process the contaminating "immunogenic moities" and stimulate an antibody response (Lukic et al, 1975; Golub and Weigle, 1969). The effects of these antigen preparations on cell-mediated immunity were unfortunately not addressed by these papers. It is possible however, that OVA contained in serum one hour after feeding behaves like a preparation of OVA which is not entirely deaggregated. Thus it would be tolerogenic for CMI due to deaggregation but perhaps unable to induce tolerance for antibody due to the presence of non-deaggregated OVA.

The failure of tolerance induction by OVA which was not fed, but injected into the systemic circulation and transferred one hour later with serum into recipients (Chapter 5) suggests that the tolerogen for CMI in serum after OVA feeding is almost certainly a product of intestinal function and is not produced after further systemic processing in the donor. It is of course impossible to tell from my experiments whether any further systemic processing of the tolerogen occurs in the recipients.

Previous experiments of this type, known as "biologic filtration" experiments, have shown the induction of tolerance for antibody responses in rabbits by antigen after 48 hours previous systemic filtration. In mice given antigen which had had 24 hours systemic filtration, antibody tolerance was induced by the filtered antigen which was less profound than that induced by an injection of native antigen (Frei et al, 1965). Subsequent experiments in mice succeeded in demonstrating that systemic filtration can produce a tolerogen for antibody responses (Lukic et al, 1975). However, in my experiments, serum tolerogen for CMI produced by OVA ingestion is present within

one hour of feeding and so seems not a product of systemic filtration by macrophages. In addition, the extremely rapid clearance of OVA from the circulation after feeding (Swarbrick, 1979) makes comparison with experimental systemic filtration difficult.

Without more detailed immunochemical analysis there existed only strong circumstantial evidence that the serum-borne tolerogen generated by feeding OVA, which induced antigen-specific suppression of DTH, was in fact antigen and not for instance, some other soluble factor induced by feeding which acted in an antigen-specific manner. The use of an ELISA technique confirmed the presence of OVA in the serum of OVA-fed mice (Chapter 6). This technique avoided the problems inherent with tracing radio-labelled proteins in serum discussed in the introduction to this thesis, which were pointed out in the experiments of Skogh (Skogh, 1982) and others (Udall et al, 1981). The ELISA was sensitive enough to detect nanogram quantities of OVA and, since detection was via the binding of OVA to affinity-purified anti-OVA IgG, also provided information about the antigenic structure of OVA in serum after feeding; the protein possessed antibody-binding sites or B cell recognition elements.

That this protein was in fact responsible for the tolerogenicity of serum containing ELISA-detectable OVA was confirmed by experiments where the tolerogenicity of serum from OVA-fed mice was removed after absorption with anti-OVA bound to Sepharose. These experiments illustrated that tolerogenic gut-processed OVA possessed structures which B cells could recognise via antibody. The experiments of Endres and Grey showed in vitro that suppressor T cells specific for native OVA could recognise the same sorts of antigenic determinants on the molecule as B cells (Endres and Grey, 1980 a). Their experiments

were based on the theory that suppressor T cells can be activated by antigen without a requirement for antigen processing by accessory cells. Gut-processed antigen could conceivably provide such a stimulus for suppressor T cells since the tolerance induced by serum transfer is CY-sensitive and almost certainly mediated by suppressor cells. However, there is no information available at present regarding systemic processing in recipients of gut-processed antigen. In addition, reports of MHC restriction in the activation of suppressor T cells indicate a requirement for accessory cells for antigen recognition by at least some types of suppressor cell (Reviewed, Germain and Benacerraf, 1981).

The possession of intact antibody-binding sites is in no way indicative of an intact molecule and so the gel-filtration studies of serum containing gut-processed OVA were useful in estimating the size of the tolerogenic protein. Significant suppression of DTH was induced in recipients by the injection of a fraction of serum from OVA-fed mice (FII), which contained proteins in the same molecular weight range as native OVA and which also contained ELISA-detectable OVA. Of all the serum fractions tested, only in FII could OVA be detected by ELISA. These results suggest that the suppressive effect of serum from OVA-fed mice is due to gut-processed OVA which is very similar to native OVA in size and in its recognition by antibody. It is however, extremely unlikely that the tolerogen is native OVA, even in deaggregated form, since my experiments described previously showed that native and deaggregated OVA both failed to reproduce the effect of gut-processed OVA in vivo.

What then, is the molecular nature of gut-processed OVA? The answer is beyond the scope of this thesis but my experiments have

opened up areas for the further investigation of this subject:

The suppressive serum fraction, FII, contained proteins within the molecular weight range of 100,000 to 40,000 approximately. Without using a more sensitive technique for the accurate resolution of proteins and peptides such as high pressure liquid chromatography (HPLC) it is impossible to pinpoint the exact molecular size of the tolerogen. Further analysis of serum using HPLC techniques should be able to reveal the presence of any subtle differences in molecular weight between the tolerogenic OVA and native OVA.

Recent work by Streicher *et al* (1984) proposes that antigen processing of some proteins involves the unfolding of at least part of the molecule and exposition of hydrophobic areas for T cell recognition. It would be exciting to test this hypothesis in an *in vivo* situation by studying the relative hydrophobicities of gut-processed OVA and native OVA.

Other work in the past, and also very recently has described the presence of both suppressor epitopes (Sercarz *et al*, 1978; Adorini *et al* 1979), and immunodominant epitopes (Berkower *et al*, 1984) consisting of as few as 17 amino acids (Shimonkevitz *et al*, 1984), on globular protein antigens for T cell recognition. Removal of such epitopes alters the immune response to the native molecule. Further study of gut-processed OVA might reveal either the exposition of a suppressor epitope on the external surface of the molecule, or the removal of an immunodominant epitope, or even both events occurring simultaneously.

Another aspect of gut-processed OVA meriting further study is the overall surface charge of the molecule. OVA which has been absorbed across the intestinal mucosa has had to interact with epithelial cell

membranes. Since lymphocyte receptors classically bind to antigens via non-covalent interactions, an altered surface charge occurring as a result of absorption may influence the subsequent immune recognition of antigen.

Further study of this tolerogenic molecule would be of value towards our understanding of the regulation of immunity by antigen feeding and also of immune recognition and regulation in general.

9.4 The Importance of Intestinal Function and GALT in the Induction of Oral Tolerance

The experiments described in Chapter 8 revealed the importance of the gut in generating tolerogenic OVA in serum after feeding. Damage to the gut inflicted by irradiation prevented the production of the tolerogen. OVA was detected in the serum of irradiated, OVA-fed mice but was not tolerogenic in nature when transferred into recipients. In addition, the systemic antigen dose in transferred serum was not related to the induction of tolerance in recipients. The absence of tolerogenic OVA in serum was noted when the intestinal crypts were in a state of either hypoplasia at 2 days after irradiation, or hyperplasia which was seen 5 days after irradiation, and therefore seems not related to a specific type of abnormality in the gut.

One possible explanation for the loss of tolerogen production in irradiated mice is that whole-body iradiation impairs proteolysis of OVA in the intestine. The experiments of Strobel showed that OVA which was administered to mice via the colon as an enema, and therefore removed from the influence of gastric and pancreatic enzymes, was effective in inducing systemic tolerance (Strobel, 1984). Proteolysis may therefore be relatively unimportant in the production

of tolerogenic OVA by the gut.

Another reason for the loss of tolerance induction following serum transfer from animals given whole-body irradiation, could be the impaired clearance of immunogenic moities of OVA from the circulation, due to either hepatic or renal dysfunction. These possiblities cannot be excluded on the basis of experimental evidence in this thesis and further work, perhaps involving the isolated irradiation of the intestine should be able to clarify this situation.

The production of tolerogenic OVA by the gut was restored by an injection of normal spleen cells. The experiments of Hanson on similarly treated mice had shown that tolerance was not induced in irradiated and reconstituted mice at 2 days after irradiation and spleen cell transfer but was restored by 5 days (D. Hanson, personal communication 1982). My results show that the intestine is not impaired in its ability to produce a tolerogen at either of these time points after irradiation and reconstitution.

Tolerogen production was restored whilst the animals were still recovering from the effects of irradiation such as weight loss and dehydration but had approaching normal numbers of jejunal intraepithelial lymphocytes. This is an intriguing observation which will require much more work before an explanation is available. The apparent recovery of the numbers of IELs after an injection of spleen cells, with the concomitant recovery of tolerogen production is curious and several potential explanations can be put forward. Either, that IELs are relevant to gut antigen processing or, perhaps more likely, that the restored IEL count is coincidental, and the re-population of lymphoid cells in GALT permits the development of IELs which have no influence on gut processing but merely indicate the

restoration of some gut function including antigen processing. In this case, the lymphoid compartment of the gut may be necessary for antigen processing either by direct interaction with antigen or by some secondary function in the maintenance of mucosal integrity. From this point of view, phagocytic cells may be of importance in repairing the radiation-damaged intestine and clearing lymphatic channels.

In any event, it can be concluded that the production of tolerogenic OVA after feeding seems to be a facet of normal gut function and may be dependent on lymphocytes.

One of the primary effects of irradiation is damage to cell membranes. This has been observed in lymphocytes which were unable to traffic normally following irradiation but which were still capable of expressing effector function (Kettman and Mathews, 1976). If lympho-epithelial interactions are relevant in the gut-processing of OVA this could well be impaired by irradiation and restored by the injection of healthy lymphoid cells.

9.5 Concluding Remarks

The work described in this thesis has shown that in adult mice, protein uptake by the intestine is of profound immunological significance.

Study of an end-product of the ingestion and absorption of a soluble, globular protein antigen, ovalbumin, has been a useful aid in visualizing interaction of the immune system with a normal physiological sytem, that of the intestine, and has provided information about the regulation of immunity as a consequence of protein antigen ingestion.

Interaction between the gut and the immune system is potentially of enormous importance to the well-being of an individual. Further

study of the immunological processes related to the intestine and of their disruption for reasons either genetic, immunological, or physiological can only improve our understanding of disease processes in the intestine.

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